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SOME OBSERVATIONS ON A DISEASE OF ZEBU CATTLE ASSOCIATED WITH INFESTATION BY THE HOOKWORM, *BUNOSTOMUM PHLEBOTOMUM*

By

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INTRODUCTION

From the beginning of this century reports of the pathogenicity of *Bunostomum phlebotomum* in cattle have appeared in the literature.

In the United States, Stiles (1901) found the parasite to be present in 50 per cent. of cattle, Dawson (1906) associated it with "salt-sick" in Florida and believed it to be the cause of the condition, and Conradi and Barnette (1908) described a number of cases of infestation in South Carolina, all of which were characterised by pale watery blood and pale flabby muscles. The Committee on parasitic diseases of the American Veterinary Medical Association (1932) reported that *B. phlebotomum* occurred in the East, South, and Middle Western States and was responsible for severe unthriftiness and death among calves. In Italy, De Gasperi (1912) described a disease in four calves infested by the parasite. The symptoms observed were extreme anaemia and emaciation of insidious onset, pica, grinding of the teeth, oedema of the thorax and abdomen, marked venous pulse, sunken eyes, pale mucosae, and a period of coma before death. Some animals also showed nasal discharge and diarrhoea. Autopsy lesions included pale, watery muscles, generalised oedema and fatty degeneration of the heart muscle and kidneys. Part of the yellow bone marrow was changed to the red variety and the duodenum showed catarrhal inflammation and ecchymoses at the sites of attachment of *B. phlebotomum*. Boynton and Wharton (1916) in the Philippine Islands described severe anaemia and progressive emaciation in cattle on low-lying pastures; autopsy revealed pallor of all the visceral organs and infestation of the duodenum by *B. phlebotomum*. Reïsinger (1916, 1924) in Austria described a similar condition which was associated with petechial haemorrhages in the duodenum caused by several hundred hookworms. It has also been observed by Csontos and Pataki (1918) in Hungary, Sprehn (1925) in Germany, Bedel (1927) in France, Duschanek (1909) in Bohemia, Szigetwary (1931) in Austria, Poisson and Buck (1938) in Madagascar, Cassamagnaghi (1945) in Uruguay, and Roberts (1939) in Queensland. The Hungarian workers reported that the faeces showed a positive benzidine test for blood. The urine was normal. Haemoglobin was 25 per cent., (Sahli); erythrocytes, 3,305,000 per c.mm.; leucocytes 7,275 per c.mm. Differential leucocyte counts showed lymphocytes, 60 per cent.; monocytes, 3·9 per cent.; neutrophils, 33·6 per cent.; eosinophils, 2·1 per cent.; basophils 0·4 per cent. Cassamagnaghi found that the erythrocyte count was reduced by 70 per cent. and that the neutrophil leucocytes were increased by 17 per cent. while Roberts found that the erythrocyte count was reduced by 75 per cent.

HISTORY

The present paper records some observations made at the Veterinary Laboratory, Northern Nigeria, upon a disease of yearling zebu cattle. The precise aetiology was obscure but the incidence appeared

to be related to the degree of infestation by *B. phlebotomum*, and the symptoms and lesions resembled those reported by the above-mentioned observers.

The disease occurs at the Veterinary Stock Farm at Vom, Northern Nigeria, where a herd of about 700 head, including milking cows, calves and store yearlings, is maintained on 2,500 acres of grassy downland.

The dry season extends from November to April and the wet season from May to October. During the dry season, owing to the deterioration of the pastures, the cattle become very thin, but most of them survive this adverse period and regain condition at the onset of the rains in April. During the dry season the diet of the stock-farm cattle is supplemented with a daily ration of cotton seed and hay. A certain number of animals, however, show signs of progressive anaemia, extreme cachexia and exhaustion, usually terminating fatally in a few weeks. The condition appears to have occurred for a number of years, beginning to appear in January and disappearing when the rains start. The disease was first seen by the writer in the dry season of 1943, and it was noticed that in each case there was a heavy infestation with *B. phlebotomum*, the bovine hookworm. By comparison with 20 healthy cattle slaughtered in the same district at the same time of the year, it was found that the average hookworm burden of the diseased cattle was about five times greater than normal. Furthermore, the symptoms and lesions shown by these animals bore resemblances to those attributed to *B. phlebotomum* by various other workers mentioned above. Further investigations were, therefore, carried out to determine whether this parasite might be involved in the aetiology. At the end of the wet season of 1943, 40 calves, six to 12 months of age, were chosen at random and the faeces were examined for the presence of the eggs of *B. phlebotomum*. In January, 1944, the faeces were re-examined, all those animals which had shown hookworm eggs on both occasions and all those which had shown them on neither occasion were noted and their progress during the dry season was observed. The history of these two groups of animals at the end of the dry season of 1944 is shown in Table I.

It will be noted that seven out of 12 animals in the first group died of anaemia while all of the ten animals in the second group survived. The average haemoglobin of Group I fell by 29 per cent. to 75 per cent. between September, 1943, and January, 1944, while that in Group II fell by 19 per cent. to 100 per cent. The seven animals in Group I died between January and April, 1944, all showing very similar symptoms and lesions.

The description which now follows is based on observations made upon these seven animals. In the case of four of them, when it appeared to be quite certain that death would shortly occur, their throats were cut to ensure that histological material would be free from *post-mortem* changes. Apart from these seven animals, several

TABLE I

SHOWING INCIDENCE OF ANAEMIA IN TWO GROUPS OF CATTLE. GROUP I SHOWED EGGS OF *B. phlebotomum* IN THE FAECES BOTH IN SEPTEMBER, 1943 AND JANUARY, 1944. GROUP II SHOWED EGGS ON NEITHER OCCASION

Animal	*Haemoglobin per cent., September*, 1943	*Haemoglobin per cent., January*, 1944	*History at end of dry season, March*, 1944	*Total hookworm burdens*
		GROUP I		
1	105	65	Died*	600
2	90	70	„	800
3	95	35	„	500
4	100	95	„	Not counted
5	90	30	„	700
6	120	40	„	600
7	100	80	„	400
8	110	85	Unknown†	—
9	95	85	„ †	—
10	100	70	„ †	—
11	130	100	„ †	—
12	120	110	Healthy‡	85
Average	104	75		
		GROUP II		
1	110	90	Unknown†	
2	110	120	Healthy	
3	100	100	„	
4	140	90	„	
5	140	110	„	
6	120	85	„	
7	100	95	„	
8	120	115	„	
9	115	100	„	
10	135	100	„	
	119	100		

* These seven animals all showed the typical symptoms and lesions described in this paper.

† These five animals were used in another experiment and their subsequent progress is unknown.

‡ This animal was slaughtered for comparison with the seven which died. The carcase was well covered with fat, the muscles were dark red and well bound together with fatty connective tissue. The heart, blood, liver, kidneys and bone marrow were normal.

other yearlings at the stock farm became ill and died, and although in most cases blood examinations were not carried out before death it was evident from their carcases that they had been suffering from a similar disease.

SYMPTOMS

The first signs of the onset of the disease were that the animal was losing weight, was falling behind the others as they grazed, and that the coat had become rough and lustreless. The animal became gradually thinner and weaker ; the vertebral spines, the ribs and the bones of the pelvis became very prominent, and the eyes were sunk within their orbits. It would stand with its head stretched

forward, grinding its teeth, its legs quivering as though it experienced difficulty in keeping upright. After a time it would go down and seldom rise again unless assisted. It would eat food offered in the hand, but would make no attempt to feed from a bucket. It lay on its breast, with its head turned round and resting on its left shoulder. Finally, after a period of illness of two to four weeks, the animal collapsed and died. At the exhaustion stage the pulse was weak and easily compressed, but the jugular pulse was very apparent. The heart beat was 60 to 70 per minute and the respiration, carried out in two distinct phases, 20 to 25 per minute. The buccal and conjunctival mucosae became very pale and of a grey tinge; there was no icterus. There was no rise of body temperature. The faeces were dark green on being passed but quickly turned black; no blood was detectable by the benzidine test, but they were covered with a film of mucus. The urine, which was of normal colour and consistence, contained no casts nor abnormal chemical constituents.

Post-mortem OBSERVATIONS

In four cases the animals were killed *in extremis*. The blood was pale, watery and clotted slowly. During flaying, the intense whiteness of the whole carcase and the complete absence of any subcutaneous fat were very striking. The subcutaneous tissues appeared as though water had been poured over them and gelatinous lumps of water-logged tissues could be lifted away from the lumbar and sacral fascia. The muscles were pale-pink, with no fat between them. The joints, especially the hip, were swollen with a clear fluid, but this was not noticeable in life. The heart, after removal, lay characteristically flattened out; its surface was marked by pink and yellow stripes and occasionally *Cysticercus bovis* and small gritty white nodules were observed in the myocardium. The finger could be pushed through its wall. The coronary grooves were filled with white jelly. The spleen was not enlarged but the capsule and the trabeculae were more prominent than usual. The liver was of normal size but of variable colour, varying from a pale grey to a dirty yellow; the substance was soft and friable. The kidneys were a brownish yellow colour, and the renal pelvis contained a loose clear jelly. There was no perirenal fat. The pericardial and abdominal cavities contained an abnormal quantity of serous fluid. The marrow cavity of the femur was filled with a transparent jelly containing islands of red marrow, which resembled red-currant jelly in consistence. No normal yellow marrow was observed.

The alimentary canal was normal except for the duodenum which in each case was studded with small red spots to most of which was attached a hookworm, either *B. phlebotomum* or, more rarely, *Gaigeria pachyscelis*. They were congregated just behind the pylorus, extending in distribution down the duodenum for a few feet. Occasionally they occurred in the jejunum and in heavy infections, also in the ileum. The number in each carcase is shown in

Table I. The abomasum and intestine contained several other species of nematode, namely, *Haemonchus contortus*, *Trichostrongylus colubriformis*, *Cooperia pectinata*, *Cooperia punctata*, *Trichuris globulosa* and *Oesophagostomum radiatum*, but none of these was present in abnormal numbers and no alteration of the mucosa due to their presence was observed.

PATHOLOGICAL HISTOLOGY

Kidney. The glomeruli showed an increased cellularity with, in many cases, partial or complete obliteration of their structure, the space within Bowman's capsule being also obliterated. The invading cells had large, irregularly shaped, fairly deeply staining nuclei with one to four nucleoli, and were possibly endothelial in origin. These cells within the emerging capillaries gave a star-shaped appearance to the glomerular tuft. There were no proliferations of the cells of the capsule. The tubules contained no blood and were usually patent, although the free border of the cells was irregular.

Liver. Variations of liver involvement between little or no departure from the normal and marked fatty degeneration, were observed. A fine pericellular cirrhosis was evident and considerable degeneration appeared to have taken place in the hepatic cells, their nuclei being pyknotic and the cytoplasm staining with very varying intensity. Osmic acid revealed that the centre of the lobule was most affected by this fatty change. In most cases the capsule was thicker than normal.

Heart. Some cases showed little change in the heart muscle cells, the cross striations were clearly defined and the cytoplasm evenly stained. In other cases the cytoplasm was coarser, and while the longitudinal striations were more clearly defined, the cross striations were difficult to discern. The cytoplasm of the cells in one case showed small unstained circular spaces, suggesting the presence of fat, while another suggested more extensive fatty infiltration with various distinct circular spaces among the cells.

Bone marrow. The erythropoietic islands were scarce and separated by large areas of non-cellular material giving the impression that the rate at which erythropoiesis was proceeding must be considerably reduced. The occurrence of many erythroblasts and normoblasts within the islands themselves showed, however, that the process was not entirely suppressed. Among the numerous granular leucocytes and myelocytes present were some eosinophils in spite of the fact that none of them was seen in the blood smears.

THE BLOOD PICTURE

The blood of every case was examined very carefully for pathogenic organisms. Two cases showed *Theileria mutans*, but no other living organisms were observed in smears of blood or of spleen and bone marrow. Blood and emulsions of spleen and bone marrow

injected intravenously into healthy calves failed to produce any effect on them. The absence of blood protozoa was to be expected, as these animals were dipped regularly, and trypanosomes are seldom or never found in cattle residing permanently on the Nigerian Plateau.

The blood picture showed a gradually developing anaemia running concurrently with the deterioration of the body condition. Table II, the record of animal No. 1 (Table I), shows the typical course of events as the dry season advanced. This animal died at the end of February.

TABLE II

EXAMINATION OF BLOOD

	September, 1943	*January*, 1944	*February*, 1944
Haemoglobin per cent. ...	105	65	30
Erythrocytes, mil. per c.mm.	9·2	4·4	2·5
Colour index	0·85	1·10	0·87
Condition	Good	Poor	Dying

The blood picture in four animals which were kept under observation before being killed *in extremis* is given in Table III.

TABLE III

THE BLOOD VALUES OF FOUR MORIBUND ANIMALS IMMEDIATELY BEFORE SLAUGHTER

					Normal values
Erythrocytes, mil. per c.mm.	2·5	2·5	2·1	1·7	7·0–12·0
Mean corpuscular diameter (microns.)	5·0	5·1	5·2	6·0	5·0
Range	3·5–7·0	4·0–7·5	4·0–7·0	4·5–8·0	4·0–6·0
Reticulocytes	0	0	0	0	0
Haemoglobin, per cent. ...	35	30	35	40	90–140
Colour index	1·02	0·87	1·25	1·76	—
Leucocytes, per c.mm. ...	7,700	10,900	4,400	7,500	8,000
Leucocytes :—					
Neutrophil, per cent					
Non-lobulated ...	6	13	Not estimated	11	0–4
Lobulated	79	61	„	51	4–48
Eosinophils, per cent. ...	0	0	„	0	0–10
Basophils „ ...	0	0	„	0	0–2
Lymphocytes „ ...	11	24	„	41	41–96
Monocytes „ ...	4	2	„	3	0–10

Erythrocytes. The corpuscles were of normal shape in all cases, no sickle forms being observed. Their appearance after Giemsa staining was normal. Reticulocytes were not seen after vital staining with brilliant cresyl blue, they were also not observed in the blood of healthy cattle. Jolly bodies and normoblasts were not observed.

The number of erythrocytes per c.mm. of blood in ten healthy yearlings was found to vary between seven and 12 million, the average number being 8,500,000. Tables II and III show that a considerable decrease in erythrocytes occurred in the diseased animals.

Blood platelets. Many of the blood platelets were larger and more globular than usual. They had a clearly defined margin, staining red with Giemsa stain, and contained several red granules in the centre. Some of these platelets measured 4 microns in diameter.

Leucocytes. Total leucocytes per c.mm. of blood were within normal range. Differential leucocyte counts showed an increase above normal of the total neutrophils and the proportion of non-lobulated neutrophils. Eosinophils were absent from the smears. (See Table III.)

Haemoglobin. This was estimated by the Sahli method. Unfortunately no instrument giving an absolute standard of g. per 100 ml. was obtainable and the results are purely comparative. The average for 40 healthy yearlings was 115 per cent.

Colour Index. As there were no absolute figures for haemoglobin, the M.C.H. could not be calculated; a colour index was therefore devised, based on the mean values obtained from healthy animals, giving the following equation :—

$$\frac{\text{115 per cent. haemoglobin}}{\text{8·5 million R.B.C.s} \times \text{Constant 13·5}} = \text{C.I. 1}$$

It can be seen from Table III that affected animals showed a wide variation in colour index.

Mean Corpuscular Diameter. This was estimated by measurement with a micrometer eyepiece. It can be seen from Table III that the M.C.D. was normal or slightly greater than normal, and that some degree of anisocytosis was present.

Discussion on Anaemia

To confirm that the bone marrow in affected animals was definitely abnormal, observations were carried out on the bone marrow of apparently healthy animals. This work (unpublished) showed that in normal cattle the red marrow was replaced by yellow marrow, and that, by nine months the red marrow was restricted to a peripheral layer in the medullary cavity, a cap at each end of this cavity, and to the cancellous epiphyses at each end of the femur. At 15 months red marrow persisted only in the upper epiphysis and in the arch-like cap at the top of the medullary cavity. The spread of the yellow marrow was regular and no centres of red marrow were left surrounded.

To compare the diseased marrow with that occurring in chronic post-haemorrhagic anaemia, 500 ml. of blood was removed each day from a heifer known to be free from hookworm. This was continued for a period of two months until the red count had fallen to

3,000,000 per c.mm. *Post-mortem* examination showed that the yellow marrow in the femur remained firm and fatty and that the red marrow had increased in extent but remained normal in appearance. Furthermore, the blood of this animal showed many reticulocytes at the time it was killed.

The bone marrow of the affected animals suggested an aplasia of erythropoiesis and this is confirmed by the lack of regenerative changes in the circulating erythrocytes even where the anaemia is severe. The condition is, however, exceptional in that the granular leucocytes were present in the blood in large numbers and with a high proportion of non-lobulated neutrophils. This shows that although erythropoiesis was much below normal, leucopoiesis was still active.

The absence of icterus, the normal shape of the corpuscles and the absence of abnormal deposits of haemosiderin in the liver and spleen, are evidence against abnormal destruction of circulating erythrocytes. It seems, therefore, that the animals died of a progressive aplastic anaemia.

DISCUSSION

Although it appears that the anaemia was due to dysfunction of the bone marrow, rather than to the disruption or loss of erythrocytes, it is not possible with the available evidence to explain its aetiology.

The occurrence of the disease at a time when food is very scarce suggests a nutritive deficiency as the cause, but the following observations are against the assumption that it is the sole cause.

(*a*) Only a certain proportion of the animals at the farm are affected, others remaining quite healthy. (See Fig. 1.)

(*b*) Nomadic cattle on similar but unlimited pastureland do not appear to suffer from the condition and regular estimation of the haemoglobin values of these animals throughout the year showed that the haemoglobin values are higher at this time of year than during the rainy season. (Sprent, 1946.)

(*c*) Hookworm-free animals confined in pens and kept on food similar to that available to the stock farm cattle in the dry season remained perfectly healthy.

(*d*) The addition of cotton seed to the diet of affected animals did not improve their condition and as many died on a diet of cotton seed, hay and pasture as on a diet of hay and pasture only.

(*e*) Administration of 200 c.c. per day of a solution containing 5 per cent. ferrous sulphate, 1 per cent. copper sulphate and 0·005 per cent. cobalt sulphate failed to improve the condition or the blood picture of affected animals.

In the introduction attention was drawn to the striking correlation between the incidence of the disease and infestation by *B. phlebotomum*. Out of 12 animals whose faeces contained eggs of *B. phlebotomum*, both in September, 1943, and January, 1944, seven

died with typical anaemic symptoms between January and April, while of 12 animals whose faeces contained eggs on neither occasion, none died. (See Table I.) The average number of *female* hookworms in these affected animals was 385, while in one animal which remained healthy and was killed for observation (Table I, Group 1, No. 12) only 54 female hookworms were counted.

In the examination of six other animals which appeared to be suffering from the same condition, the relationship between the degree of infestation by *B. phlebotomum* and the appearance of typical symptoms and lesions was not so apparent. Two animals which had evidently become anaemic contained only 42 and 59 female hookworms each and it is difficult to understand how so few could cause disease, although it has been stated by Mönnig (1938) that 24 specimens of *Gaigeria pachyscelis* can cause death in the sheep. It has been shown (Sprent, in press) that in the locality where this disease occurs in Nigeria the hookworm burden of native-owned nomadic cattle, which enjoy more or less unlimited grazing range, fluctuates throughout the year and that it is greatest during the last months of the dry season. Similar conditions of infestation are also likely to obtain under stock farm conditions, but whereas the average female hookworm burden of nomadic cattle for the months of January, February and March, 1944, was found to be 56, and no more than 200 females were ever seen in one animal, the average female burden in all stock farm animals observed at autopsy throughout these three months was 141 and in some of the diseased animals was over 500. It is thus evident that stock farm conditions predispose to hookworm infections and that the period of maximum hookworm burden coincides both with the period of food shortage and the period of incidence of the disease described in this paper.

Fourie (1942) has described outbreaks of acute helminthiasis in cattle under climatic conditions which appear to be very similar to those in Northern Nigeria. The outbreaks occurred at the middle or end of the dry season when the pastures were at their poorest.

However, the evidence which supports the conclusion that *B. phlebotomum* is the cause of, or plays an important part in the genesis of the condition described in this paper is entirely circumstantial. Firstly, the symptoms and lesions resemble those reported by other observers who attribute pathological significance to the parasite; secondly, the incidence of the disease bears some relationship to infestation with the parasite; and, thirdly, the period of incidence of the disease coincides with the period at which hookworm infestation is greatest. The writer has studied the problem from several different angles without, however, obtaining more convincing evidence. Treatment of affected animals with anthelmintic drugs with a view to ascertaining the effect of removal of the parasites yielded no information of value as none of the drugs used displayed the necessary action. Attempts to reproduce the typical anaemic condition in cattle by infesting them experimentally with

B. phlebotomum miscarried, as only a very light degree of infestation could be attained. A third line of investigation was undertaken involving a study of the activities of *B. phlebotomum* in the duodenum. It was felt that the evidence incriminating the worm would be more impressive if it could be shown, for example, that it damages the intestinal wall sufficiently to cause absorption to be deranged, or that it produces a substance which on absorption acts by inhibiting erythropoiesis. So far, however, no definite indication as to the mode of action of the worm has resulted from this study. The work done in connection with this and the preceding investigations will be recorded more fully elsewhere.

SUMMARY

A disease of Zebu cattle, occurring in the dry season (January to April) in Northern Nigeria is described. It is characterised by progressive emaciation and exhaustion, with the development of an aplastic anaemia. The heart and liver showed signs of cellular degeneration and the presence of abnormal fat. In its incidence the disease bears some relationship to the degree of infestation by the bovine hookworm, *B. phlebotomum*, and it occurs at the time of year when the hookworm burden of nomadic cattle was found to be maximal. Furthermore, the symptoms and lesions bear a general resemblance to those described by other observers in cases of heavy infestation by this parasite. The evidence is, however, circumstantial and until more is known of the activities of the parasite in the duodenum the aetiology of this condition must be left undecided.

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FIG. 1.

Two yearling Zebu cattle born in the same week. The smaller animal whose faeces contained 2,000 eggs of *B. phlebotomum* per g. shows symptoms of the disease. The larger animal's faeces contained no eggs. Both were kept under identical conditions.

IMMUNISATION AGAINST FOWL PLAGUE WITH CRYSTAL-VIOLET VACCINE

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INTRODUCTION

Since fowl plague was first described by Perroncito (1878) and later differentiated from fowl cholera by Rivolta and Delprato (1880), this disease has been the subject of intensive study by workers in virus research. This interest has been largely stimulated by the relative stability of the causal virus, its infectivity and the ease with which it can be isolated and cultivated. Fowls which survive an attack of the disease develop a strong immunity and consequently much attention has been paid to its immunological aspect and considerable effort expended in evolving suitable vaccines. For this reason, the relevant literature is necessarily extensive and can therefore be referred to only briefly.

A variety of means have been adopted in order to modify the virus. Heat treated virus has been tried by Maggiora and Valenti (1904) Jouan and Staub (1920), Staub (1926), Gerlach and Michalka (1926), Doyle (1927) and others but with only slight success. Lagrange (1932), Kondo and Nakamura (1934), Hallauer (1936), Nakamura *et al.* (1937) and Coronel (1939) have described experiments in which attenuating agents such as formalin, chloroform, etc., usually in conjunction with heat or desiccation, were used, and though good results were claimed by some, the majority of vaccines thus produced were ineffective. Todd (1928) stated that a phenol-glycerine vaccine attenuated at room temperature gave satisfactory results, but similar vaccines tested by Purchase (1930) and Beaudette *et al.* (1934) were found to be useless.

Other authors such as Plotz (1933, 1937), Hallauer (1934, 1939), Schmidt and Ørskov (1935), Weineck (1938) and Kraneveld and Nasoetion (1939) have passaged the virus in tissue culture or through susceptible animals, and though in some cases it was sufficiently modified by these means to be comparatively avirulent, its antigenic value was low. Otherwise, the effect of passage was merely to leave the virus unaltered or to exalt its virulence considerably.

Lépine *et al.* (1936) subjected the virus to enormous pressures, and Levin and Lominski (1936) exposed it to X-ray irradiation but vaccines evolved by these means were of uncertain value. Other unorthodox methods were tried by Ørskov and Schmidt (1935) and by Bijl and van der Schaaf (1936) but with little success.

It is clear from the literature that none of the recognised means of virus modification provides a reliable immunising agent against fowl plague and consequently, prompted by the success reported by McBryde and Cole (1936) in developing a crystal-violet vaccine against swine fever and by an experiment on Newcastle disease virus

with this dye by Iyer and Dobson (1941), the following work was planned.

1. The passage of fowl plague virus through egg-embryos to ascertain if it became more virulent, and if so, to what extent.

2. The use of virus obtained from infected egg-embryos, in combination with crystal-violet dye and heat, and the testing of its immunising properties in fowls.

EFFECT ON THE VIRUS OF PASSAGE THROUGH EGG-EMBRYOS

Technique. A known strain of fowl plague virus was obtained in the form of infected liver and spleen : two fowls each inoculated subcutaneously with 1 ml. of a suspension of these tissues died in 48 hours, both showing lesions of the disease on *post-mortem* examination. Portions of liver tissue from these fowls were finely ground and suspended in saline or fowl plasma, passed through glass wool to remove coarse particles and filtered through either Seitz or " gradocol " filters (A.P.D. 0·81μ). The presence of virus in the filtrates was confirmed by inoculating 1 ml. into normal fowls.

Using Burnet's technique, six ten-day-old egg-embryos were inoculated by pipetting 0·05 ml. of filtrate on to each chorio-allantoic membrane and sealing in the usual way with vaseline-paraffin wax. In 48 hours, all the embryos were dead and passage to a further six ten-day-old embryos was effected by inoculating them in a similar manner with 0·05 ml. of an inoculum, prepared by diluting 0·1 ml. of infected allantoic fluid in 5 ml. of normal saline. These six embryos also died within 48 hours. The virus was subsequently passaged 200 times through egg-embryos, the ages of which at the time of inoculating varied between eight and eleven days.

Tests of virulence were carried out at the 1st, 15th, 30th, 45th, 60th, 120th, 150th and 200th passage through egg-embryos. A 10 per cent. tissue suspension by weight in saline was prepared and a series of dilutions in normal saline was injected in doses of 5 ml. into normal fowls of more or less uniform weight and age.

TABLE I

TITRATION OF PASSAGED VIRUS

Dilutions of 10 per cent. embryo suspension	*Virus Passage*							
	1st	*15th*	*30th*	*45th*	*60th*	*120th*	*150th*	*200th*
10^{-4}	x	x	x	x	x	x	x	x
10^{-5}	xx	xx	xx	xx				xx
10^{-6}	oo	xx	xx	xo	xx	xx	xx	xx
$2x10^{-6}$					xx			
$3x10^{-6}$					xx		oo	xx
$4x10^{-6}$					xx			
$5x10^{-6}$					xx	xx	oo	oo
10^{-7}	oo	oo	oo	xx	xx	oo	oo	
$2x10^{-7}$						oo		

KEY : x = Fowl died following inoculation.
o = Fowl survived inoculation.

It will be observed that the virus appears to have been considerably exalted in virulence by passage and to have reached its maximum

at the 45th/60th passage but subsequently decreased in virulence, becoming more or less constant. It is difficult to titrate accurately virus in dilutions of 10^{-6} or more as minute errors in the initial stages of preparations must later become proportionately exaggerated and, furthermore, the dilution phenomenon described by Todd (1928) may give rise to confusing results. However, it seems evident that on rapid passage through egg-embryos, fowl plague virus became greatly exalted in virulence and that infected embryos from about the 45th/60th passage provided a rich source of virus.

CRYSTAL-VIOLET VACCINE

In conjunction with tests on passaged virus, attempts were made to produce a vaccine from virus obtained from infected eggs using crystal-violet dye.

A 10 per cent. embryo suspension from a 30th passage was mixed immediately after preparation with a 3 per cent. aqueous solution of disodium phosphate and a 0·5 per cent. aqueous solution of crystal-violet (Dupont) in the proportions of 8 : 1 : 1 respectively. This mixture (Batch 1) was shaken, incubated for 72 hours at 37° C. and then inoculated subcutaneously into six fowls, each receiving 5 ml. These fowls, none of which showed any symptoms, were injected subcutaneously 22 days later, together with two unvaccinated control birds, with a virus suspension of known virulence. None of the vaccinated birds showed a reaction to this test dose but the two controls died within 48 hours. A similar vaccine was prepared in exactly the same way but instead of embryo tissue, liver and spleen, obtained from a fowl which had died of an acute attack of the disease following inoculation with egg-passaged virus, was used. Each of three fowls inoculated with 5 ml. of this vaccine (Batch 2) survived a test dose of virus 36 days later, an unvaccinated control dying in 24 hours. These results are summarised in Table II.

TABLE II

GROUP 1.—IMMUNISING PROPERTIES OF CRYSTAL-VIOLET VACCINE

Vaccination			*Immunity Test*		
Number of Fowls used	*Vaccine Batch*	*Dose and Route*	*Test Dose*	*Interval*	*Result*
6	1	5 ml. subcut.	10^6 MLD. (Approx.)	22 days	All survived
3	2			36 days	
3 (unvaccinated controls)				—	Died in 1 to 2 days

Note.—All deaths reported in this and subsequent tables were caused by fowl plague except those against which a question mark is inserted. The latter indicates that none of the lesions usually associated with the disease were observed and that it was doubtful if the bird died of fowl plague.

Subsequent tests showed that vaccine incubated for only 48 hours instead of 72 hours was harmless for fowls and in preparing further batches of vaccine the length of incubation was reduced to this period.

In view of the considerable immunising powers shown by crystal-violet vaccine, further tests were planned by which it was hoped to gain some information on the following points. The minimum immunising dose; the time taken to establish immunity; the duration of immunity; the keeping qualities of the vaccine.

No other trials were carried out with vaccine prepared from infected liver and spleen, as egg-embryos presented a cheaper, more convenient and more sterile source of virus. In order that an adequate supply of passaged virus would be continually available, several other series of virus passages in egg-embryos were commenced from time to time and batches of vaccines used in further tests were usually prepared from these subsidiary series.

The minimum immunising dose

With the intention of establishing the minimum immunising dose 15 fowls were each inoculated with vaccine prepared from a 71st passage (Batch 4) in doses ranging from 4 to 0·5 ml. After an interval of 20 days, these 15 and three unvaccinated controls were each inoculated subcutaneously with a test dose of virus. The three control birds died within 24 hours. Seven of the 15 vaccinated birds survived: of the remaining eight, seven died within seven days, showing lesions of the disease and one died in 15 days without exhibiting lesions (Group 2).

In the light of earlier successes, these results were puzzling and no explanation could be advanced until a sample of Batch 4 was retested for sterility. Up to this point, it had been customary to test the sterility of vaccine by diluting and plating and by this method Batch 4 appeared sterile, but by centrifuging 10 ml. of the vaccine, washing the deposit as free from the dye as possible and then plating, a number of colonies of Gram-negative organisms were isolated.

A further batch of vaccine was therefore prepared from infected embryos from a 24th passage (Batch 5) and more stringent tests were carried out to ensure that it was uncontaminated. Another lot of 15 fowls (Group 2a) were vaccinated with this batch in doses varying from 5 to 1 ml. After 27 days, these 15 and three unvaccinated controls were each inoculated with virus. Of the vaccinated birds, one died five days later showing lesions of visceral gout only and 14 remained well, the three controls dying within 36 hours.

The results are set out in Table III in which it will be seen that 1 ml. of vaccine was sufficient to produce immunity. In view of the simplicity and economy of preparing the vaccine, however, and because of the likelihood that antibody response was in proportion to the amount of virus given, it was decided not to reduce the dose of vaccine for succeeding tests but to maintain it at 5 ml.

TABLE III

GROUP 2a.—MINIMUM IMMUNISING DOSE

Vaccination			*Immunity Test*		
Number of Fowls used	*Vaccine Batch*	*Dose and Route*	*Test Dose*	*Interval*	*Result*
3	5	5 ml. s/c	10^6 MLD. (approx.)	27 days	2 survived; 1 died in 5 days ?
3		4 ml. s/c			All survived
3		3 ml. s/c			
3		2 ml. s/c			
3		1 ml. s/c			
3 (unvaccinated controls)				—	All died in 2 days

The time taken to establish immunity

Twenty-five fowls (Group 3) were divided into five groups of five and four of every group were each given 5 ml. of vaccine prepared from a 19th passage (Batch 7). At intervals of six to 14 days, a group of five, consisting of four vaccinated and one unvaccinated fowls, was removed from the pen and each bird was tested by the inoculation of virus. The results are shown in Table IV.

TABLE IV

GROUP 3—TIME TAKEN TO ESTABLISH IMMUNITY

Vaccination			*Immunity Test*		
Number of Fowls used	*Vaccine Batch*	*Dose and Route*	*Test Dose*	*Interval*	*Result*
4	7	5 ml. subcut.	10^6 MLD. (approx.)	6 days	All died in 2 days
4				8 days	1 died in 4 days; 3 died in 2 days
4				10 days	1 died in 9 days; 1 died in 3 days; 2 died in 2 days
4				12 days	2 died in 2 days; 1 died in 3 days; 1 survived
4				14 days	All survived
5 (unvaccinated controls)				—	All died in 2 days

It will be seen that after vaccination an interval of at least 14 days was required before a solid immunity was established. In further tests, so as to be on the safe side, no bird was tested for immunity within less than three weeks of vaccination.

The duration of immunity

For this purpose 28 fowls were each vaccinated with 5 ml. of vaccine (Batch 7). After an interval of three weeks, four birds were tested for immunity by the inoculation of virus. As these remained well and an unvaccinated control died in two days, it was assumed that the other 24 vaccinated birds were similarly protected. At intervals of 59, 91 and 151 days, groups of six were removed from the pen and their immunity tested by the subcutaneous inoculation of virus. Owing to certain circumstances, the last group of six was not tested after eight months as originally intended and is not included in Table V in which the results are summarised.

TABLE V

GROUP 4—DURATION OF IMMUNITY

Vaccination			*Immunity Test*		
Number of Fowls used	*Vaccine Batch*	*Dose and Route*	*Test Dose*	*Interval*	*Result*
*4	7	5 ml. subcut.	10^6 MLD. (approx.)	22 days	All survived
6				59 days	5 survived 1 died in 7 days (?)
6				91 days	3 survived 1 died in 10 days (?) 1 died in 6 days 1 died in 5 days
6				151 days	2 survived 1 died in 6 days (?) 1 died in 4 days 2 died in 3 days
4 (unvaccinated controls)				—	All died in 2 days

* These four birds were found to be solidly immune when again tested ten months later with approximately 10^7 MLD. of virus.

It would seem that immunity was fairly strong up to about two months after vaccination but began to fade comparatively quickly after that time. It will be noted that after 91 and 151 days, odd birds still retained their resistance to infection, and, judging by the unusually long interval between the times of administering the test dose and death, many of the remaining fowls of these groups seemed to have maintained a degree of immunity which might have been sufficient to prevent death had exposure to the virus been by contact, as occurs under natural conditions. It may be noted here that the four birds which survived their test dose of virus 22 days after vaccination were subsequently found to be solidly immune ten months later.

The keeping qualities of the vaccine

A batch of vaccine which had given good results was selected for this purpose and divided into three lots; each lot was stored under different conditions as follows.

Batch 5. A. Kept in the ice-box of the refrigerator, the average temperature of which was — 4° C.

Batch 5. B. Kept on the refrigerator shelf at an average temperature of 7° C.

Batch 5. C. Kept in a dark cupboard, the temperature of which varied between 23° and 15° C. and averaged 18° C.

The *p*H of the vaccine was tested electrometrically every two months and was found to remain uniformly steady at 8·2.

At intervals of one, three and six months, samples from the three lots were withdrawn and groups of three fowls were vaccinated with 5 ml. doses from each. After three weeks the immunity of the vaccinated birds was tested by the injection of virus.

The results in these groups, set out in Table VI, were not regular, but in view of the high proportion of survivors in the nine fowls

TABLE VI

GROUP 5—KEEPING PROPERTIES OF VACCINE

Vaccination				*Immunity Test*		
Number of Fowls used	*Age of Vaccine*	*Storage Temp.*	*Dose and Route*	*Test Dose*	*Interval*	*Result*
3		— 4° C.				All survived
3	32 days	7° C.			21 days	All survived
3		18° C.				2 survived; 1 died in 6 days (?)
3		— 4° C.				All survived
3	95 days	7° C.	5 ml. subcut.	10^8 MLD. (approx.)	20 days	2 survived; 1 died in 14 days (?)
3		18° C.				2 survived; 1 died in 7 days
3		— 4° C.				2 survived; 1 died in 6 days
3	186 days	7° C.			22 days	2 survived; 1 died in 4 days
3		18° C.				All survived
9	(unvaccinated controls)				—	All died in 2 days

treated with six months old vaccine, it seems that much of its potency was retained for that time. Furthermore, in those vaccinated birds which succumbed to the test dose, an appreciable delay was observed in the onset of death in comparison with the control birds. Bearing in mind the severity of the test dose, this result appears to indicate that some degree of protection was conferred. The influence of different temperature levels on the keeping qualities of the vaccine appeared to be negligible. It was not possible to test the potency of vaccine kept for more than six months.

DISCUSSION

From the foregoing results, it seems clear that crystal-violet vaccines prepared from infected egg-embryos had strong immunising powers, though the immunity so produced was of rather short duration. Although it was proved (Group 2a) that 1 ml. of vaccine gave ample protection against an injection of virus, a dose of 5 ml. was employed in other groups for reasons already mentioned. The most disappointing feature was the comparatively brief period of immunity to injected virus following vaccination, as was shown in Group 4, in which the standard vaccine dose of 5 ml. was used throughout, but whether the results in this group would have been more satisfactory had a larger immunising dose been employed is problematical. Purchase (1931) has also noted the short period of immunity following vaccination against the disease. The inoculation of living virus during the resistant stage apparently induces a long standing immunity, as was observed in a number of survivor birds from Group 4 which showed no reaction to injected virus when tested ten months later.

A number of writers are agreed that as a rule fowl plague vaccines do not retain their immunising properties well. Though the results obtained in Group 5 in which this aspect was studied, showed that some potency was retained up to 186 days, the evidence from this and other groups indicated that the vaccine was of greatest value when freshly prepared.

The presence of tissue in the vaccine appeared to be of some significance. Hirst *et al.* (1942), in studies on human influenza vaccines prepared from infected eggs, have recorded that the antibody response was greatest when embryo tissue and allantoic fluid were used instead of allantoic fluid alone. It is worth noting that in an uncontrolled experiment, not recorded here, a crystal-violet vaccine prepared from highly virulent allantoic fluid gave very poor results in comparison with those given by one obtained from the embryos of the same eggs.

The necessity of keeping the vaccine free from contamination is important as was shown by the results in Group 2 (not tabulated) which were vaccinated with a batch proved later to have contained Gram-negative organisms. The production of virus in fertile eggs for fowl plague vaccines, whether for treatment with formalin, phenol or other means, has the advantage of providing a ready source of sterile virus : moreover, as about 500 ml. of crystal-violet vaccine can be obtained from six 12 days old embryos, it has the additional merit of economy. By using egg-embryos more than ten days old for virus cultivation, a correspondingly greater yield of vaccine can be procured but the increased maturity of the embryos tends to make the preparation of a tissue suspension more difficult.

No attempt was made to assess the effects of a double injection of vaccine as has been done by many workers, for, though the value of the reinforcing dose in establishing a strong immunity is fully

appreciated, it was felt that the practical aspects of such a method as a means of control would present obvious difficulties. No opportunity was available to repeat some of these experiments using different strains of virus and the possibility that quite different results might have been met with had this been done is well realised.

SUMMARY

The virus of fowl plague was passaged 200 times through egg-embryos and at the 1st, 15th, 30th, 45th, 60th, 120th, 150th and 200th passages its virulence was titrated in fowls. The virulence greatly increased, reaching a maximum at the 45th/60th passage and then decreased, subsequently remaining more or less constant.

Six fowls, each vaccinated with 5 ml. of crystal-violet vaccine prepared from passaged virus in infected egg-embryos, were found to be immune when tested by the inoculation of live virus 22 days later.

Three fowls, each vaccinated with 5 ml. of crystal-violet vaccine prepared from virulent liver and spleen tissue were also found to be immune when tested 36 days later, but as infected egg-embryos provided a more satisfactory source of virus, no further tests on this type of vaccine were carried out.

Fourteen out of 15 fowls, vaccinated with doses ranging from 5 to 1 ml. (each of three fowls receiving 5, 4, 3, 2, and 1 ml. respectively) were immune when tested 27 days later. Although 1 ml. was sufficient to produce immunity, a 5 ml. dose was maintained in subsequent groups because of the probability of a greater antibody response to the larger dose.

Twenty fowls were each given 5 ml. of vaccine and were tested with virus in groups of four at intervals of six, eight, ten, 12 and 14 days later. Only those tested 14 days afterwards remained well, showing that an interval of at least two weeks after vaccination was required to establish immunity.

Eighteen fowls were tested in groups of six by the inoculation of virus at intervals of 59, 91 and 151 days respectively after vaccination. Only those tested after 59 days were immune but from the delayed onset of death in birds which died in the other vaccinated groups, it was concluded that some degree of resistance was maintained up to six months.

Three groups of nine fowls were treated with vaccine which had been kept for 32, 95 and 186 days respectively under different temperature conditions. When tested for immunity three weeks later by the inoculation of virus, 66 to 77 per cent. of each group survived and it was concluded that though vaccine retained, to a large extent, its powers of protecting against inoculated virus for at least six months, it was of greatest value when freshly prepared. The temperature at which vaccine was stored did not appear to affect its keeping properties.

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THE ESTIMATION OF PENICILLIN IN BLOOD SERUM AND MILK OF BOVINES AFTER INTRAMUSCULAR INJECTION

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INTRODUCTION

The sensitivity of most pathogenic *streptococci* and *staphylococci* to the bacteriostatic action of penicillin, both *in vitro* and *in vivo*, has been recorded by numerous workers. This report describes work undertaken to reveal whether similar organisms causing or complicating diseases of the bovine mammary gland were also sensitive to the action of penicillin. Further work was included to show whether it was possible to produce and maintain a bacteriostatic level of the drug in the milk of cattle by intramuscular injection. Finally, the effect of bovine serum on the action of penicillin was investigated.

The calcium and sodium salts of penicillin, in crude form, were available as compressed tablets, each of which was specified to contain 10,000 or 9,000 Oxford units. During the three months occupied by the experiment the tablets were stored at 5° C.

THE SENSITIVITY OF PATHOGENIC COCCI ISOLATED FROM THE BOVINE MAMMARY GLAND

The sensitivity to penicillin of 56 recently isolated strains of *Str. agalactiae* and of four coagulase-positive haemolytic strains of *Staph. aureus* (also recently isolated from acute infections of the udder) was determined as follows: One drop of a litmus milk culture of each of the strains was inoculated into each of a series of concentrations of penicillin in sterilised skimmed milk containing litmus (litmus milk). Change in colour of the litmus on incubation at 37° C. for three days was considered to indicate multiplication of the organism while no such change was regarded as evidence of inhibition. The growth of the majority of the streptococcal and staphylococcal strains, was inhibited in the particular batch of milk used, at a penicillin concentration of 0·02 Oxford unit per ml. (*i.e.*, 1 unit per 50 ml.). A few of the strains showed slight growth at this concentration, but all were inhibited at a concentration of 0·04 Oxford unit per ml. Florey and Jennings (1942) have defined the Oxford unit of penicillin as being that amount present in 50 ml. of broth which just inhibits completely the growth of a test strain of *Staph. aureus*. Thus the degree of inhibition of cocci associated with diseases of the bovine mammary gland when tested in litmus milk is uniform and of a similar order to that of the Oxford staphylococcus. Uniform sensitivity to penicillin among strains of *Str.*

agalactiae was also recorded by Brownlee (1945), who used autoclaved milk and found that in this medium 0·06 to 0·12 unit per ml. was required to inhibit the growth of 16 strains.

STABILITY OF PENICILLIN IN LITMUS MILK

According to Benedict *et al.* (1945), 50 per cent. of the penicillin in an aqueous solution at *p*H 6 is destroyed by incubation at 37° C. for four days. Occasionally in the present work, prolongation of incubation after one day resulted in the growth of some of the strains at concentrations of penicillin greater than that just permitting growth within one day, and the test was therefore read at three days. To ascertain whether this growth after the first 24 hours was caused by any destruction of the penicillin by incubation in litmus milk, its stability in this medium at various temperatures was investigated. A series of solutions in litmus milk (*p*H 6·3) containing 200, 40, 8, 1·6, 0·32, 0·064, and 0·013 Oxford units per ml. was prepared and incubated at 16°, 22°, 30°, 37° and 54° C. Two sets were prepared ; one was inoculated with a single drop of an 18-hour litmus milk culture of *Str. agalactiae* and the other was left uninoculated. The penicillin content of the solutions was tested at daily intervals by diluting them in litmus milk and inoculating with a strain of *Str. agalactiae* of which the sensitivity to penicillin was known. At temperatures up to 37° C. no appreciable loss in activity occurred within three days. At 37° C., however, rapid destruction took place after three days while incubation at 54° C. resulted in the destruction of 80 per cent. of the penicillin in litmus milk within 24 hours and of 96 per cent. within 48 hours. The presence of the comparatively small numbers of *Str. agalactiae* in the first set had no detectable effect on the stability of the drug. The results showed, therefore, that the test was satisfactory if read after three days' incubation, but that longer periods would lead to erroneous conclusions as to the amount of penicillin present.

THE ESTIMATION OF PENICILLIN IN BLOOD SERUM AND MILK

The amount of penicillin present in blood serum and milk was estimated by preparing successive 1 in 2 dilutions in litmus milk and then inoculating all dilutions with a strain of *Str. agalactiae* of known sensitivity to penicillin. Readings were made after incubation at 37° C. for three days, a definite pink colouration being taken as evidence of growth. Strain 606 selected for the purpose was maintained in cooked meat medium. Litmus milk subcultures were prepared for each assay. Growth of this strain was always inhibited at a uniform concentration of penicillin for each batch of litmus milk and penicillin. Several factors, however, were found to influence the assay when blood serum and fresh whole milk were added, and these were further investigated.

Factors affecting the estimation in blood serum. Until the recent report by Bigger (1944), it had been considered that blood serum

had no effect on the bacteriostatic action of penicillin. Bigger, however, showed that contact with serum previous to assaying reduced the bacteriostatic effect of penicillin. The degree of inactivation was proportional to the temperature, the length of period of contact and the concentration of serum. Contact with high concentrations for 24 hours produced inactivation which was negligible at refrigerator temperature, slight but definite at room temperature, and pronounced at body temperature. Some inactivation was noted after contact with high concentrations of serum at 37° C. for as short a period as three hours, but extensive inactivation was not produced until after 12 hours, while after 24 hours as much as 90 per cent. or more of penicillin was inactivated. Concentrations of serum of 50 per cent. or higher were found to be necessary for pronounced inactivation, lower concentrations being much less effective.

In the assay method employed in the present work the penicillin derived from the injected material was subjected to contact at 37° C. with concentrations of serum up to 50 per cent. for three days. Hence it was necessary to determine whether bovine serum contained an inactivating factor; if so to what extent; and if possible to devise some means of counteracting its action. Accordingly, a series of dilutions of penicillin was prepared in litmus milk containing various concentrations of fresh bovine serum up to 50 per cent. The test organism was inoculated and readings made after three days' incubation at 37° C. A typical result is shown in Table I, where incubation with over 1 per cent. and up to 10 per cent. of fresh bovine serum for 72 hours resulted in considerable inactivation of the penicillin which was directly proportional to the concentration of serum; higher amounts of serum produced less inactivation. It was thought that this finding, which is contrary to that recorded by Bigger (loc. cit.), might be due to some bacteriostatic action of the higher concentrations of serum on the growth of the test organism but no such effect could be demonstrated in control experiments without penicillin using the same sera.

The antagonistic effect of serum was duplicated when sulphanilamide was substituted for penicillin but it was found that this was not due to the same factor. Thus Table I shows that if the serum-penicillin or serum-sulphanilamide mixtures are heated to 56° C. for 30 minutes, the anti-penicillin factor is destroyed or at least inactivated whereas the anti-sulphanilamide factor (presumably para-aminobenzoic acid) is not affected. The assay of penicillin by the method suggested would therefore be reliable if the serum was heated before assay provided this treatment did not affect the penicillin. To test this point an experiment was set up in which varying amounts of penicillin from 2·5 units per ml. to 0·01 unit per ml. were added to fresh cow serum and the mixtures heated at 56° C. for 30 minutes. Each mixture was then diluted in litmus milk to

TABLE I

THE EFFECT OF FRESH AND HEATED SERUM ON THE BACTERIOSTATIC ACTIVITY OF PENICILLIN AND SULPHANILAMIDE

Concentration of Serum (%)	*Control no Penicillin*	*Pencillin in Oxford units per ml.*							*Sulphanilamide*						
		1·28	0·64	0·32	0·16	0·08	0·04	0·02	1/250	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
Fresh															
50	+	—	—	+	+	+	+	+	—	—	—	—	—	+	+
25	+	—	—	+	+	+	+	+	—	—	—	+	+	+	+
12·5	+	—	+	+	+	+	+	+	—	—	+	+	+	+	+
6·2	+	—	+	+	+	+	+	+	—	—	—	+	+	+	+
3·1	+	—	—	—	+	+	+	+	—	—	—	—	+	+	+
1·6	+	—	—	—	+	+	+	+	—	—	—	—	—	+	+
0·8	+	—	—	—	—	+	+	+	—	—	—	—	—	—	+
0·4	+	—	—	—	—	—	+	+	—	—	—	—	—	—	+
0·0	+	—	—	—	—	—	+	+	—	—	—	—	—	—	+
Heated to 56° C. for 30 mins.															
50	+	—	—	—	—	+	+	+	—	—	—	—	—	—	+
25	+	—	—	—	—	+	+	+	—	—	+	+	+	+	+
12·5	+	—	—	—	—	+	+	+	—	—	+	+	+	+	+
6·2	+	—	—	—	—	+	+	+	—	—	—	+	+	+	+
3·1	+	—	—	—	—	+	+	+	—	—	—	—	—	+	+
1·6	+	—	—	—	—	+	+	+	—	—	—	—	—	—	+
0·8	+	—	—	—	—	—	+	+	—	—	—	—	—	—	+
0·4	+	—	—	—	—	—	+	+	—	—	—	—	—	—	+
0·0	+	—	—	—	—	—	+	+	—	—	—	—	—	—	+

+ = growth of *Str. agalactiae.*
— = no apparent growth,

give the dilutions shown in Table II. As a control penicillin was dissolved in serum which had been heated, but the solution was not subjected to further heat treatment. The results show that growth of the test organism was inhibited with the heated mixtures at the same concentration as in the control and that the heat treatment had therefore no destructive effect on penicillin, even in the weakest concentration which could be tested.

The following technique was finally adopted for the assay of penicillin present in serum following intramuscular injection of the drug. Serum was obtained from blood samples as soon as clotting had occurred and heated at 56° C. for 30 minutes. Successive 1 in 2 dilutions were then made in litmus milk and inoculated with the test organism. The tubes were incubated at 37° C. for three days and examined daily for any colour change.

Factors affecting the estimation in milk. The use of raw milk for this purpose presented several difficulties; its bacteriostatic action inhibited growth of the test organism, while accidental contamination during the taking of large numbers of samples was difficult to exclude. Since many common organisms possess strong penicillinase activity, the destruction of such contaminants was essential. Boiling the milk would overcome both these difficulties provided it did not destroy the penicillin. Solutions of penicillin in both raw, full-cream milk and sterilised skimmed milk were therefore heated at 100° C. for varying periods. No destruction of penicillin could be detected within 15 minutes but after 30 minutes 50 per cent. and after 60 minutes 75 per cent. appeared to be destroyed. This agrees with the findings of Lawrence (1945), that penicillin can be boiled for three minutes without loss of activity, and of Abraham and Chain (1942) that in aqueous solution most of its activity is lost after boiling for 30 minutes.

For the assay of penicillin in milk, therefore, samples were held at 100° C. in a water bath for five minutes, cooled and successive 1 in 2 dilutions made in litmus milk. These dilutions, together with the original heated sample to which sterile litmus solution had been added, were inoculated with the test organism, incubated at 37° C. for three days and examined daily for colour change.

THE CONCENTRATION OF PENICILLIN IN BLOOD SERUM AND MILK AFTER INTRAMUSCULAR INJECTION

Although Fleming (1943) used doses of from 30,000 to 240,000 Oxford units of penicillin for man, Selbie *et al.* (1945) found that 20,000 units injected intramuscularly was sufficient to maintain a bacteriostatic level of the drug in the blood for three hours after injection. On Selbie's findings and using a body weight basis, the dose for cattle would thus be of the order of 100,000 units. This amount was injected intramuscularly into two heifers, each producing about a gallon of milk daily. The animals were milked out and blood samples were taken immediately before injection and at

TABLE II

THE EFFECT OF HEATING PENICILLIN IN SERUM AT 56° C. FOR 30 MINUTES

Original concentration of penicillin in serum before heating (Oxford units per ml.)	*Dilution of serum/penicillin mixtures in litmus milk*														*Control Unheated Penicillin*	
	1:2		1:4		1:8		1:16		1:32		1:64		1:128			
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
2·56	1·28	—	0·64	—	0·32	—	0·16	—	0·08	—	0·04	+	0·02	+	1·28	—
1·28	0·64	—	0·32	—	0·16	—	0·08	—	0·04	+	0·02	+	0·01	+	0·64	—
0·64	0·32	—	0·16	—	0·08	—	0·04	+	0·02	+	0·01	+			0·32	—
0·32	0·16	—	0·08	—	0·04	+	0·02	+	0·01	+					0·16	—
0·16	0·08	—	0·04	+	0·02	+	0·01	+							0·08	—
0·08	0·04	+	0·02	+	0·01	+									0·04	+
0·04	0·02	+	0·01	+											0·02	+

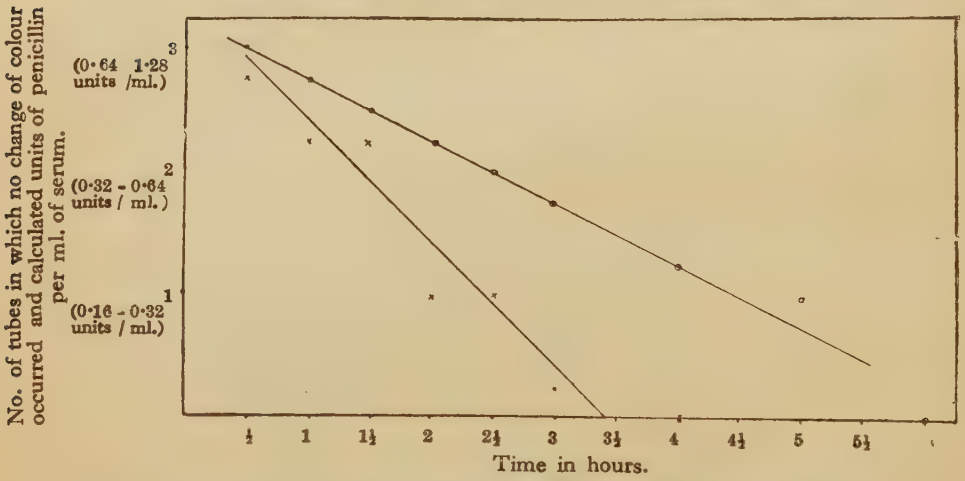
Column A = Concentration of penicillin assuming no destruction occurred due to heating.
Column B = Growth of the test organism. + = growth. — = No growth.

half-hourly intervals thereafter for five hours. The only indication of penicillin in the blood was in one sample taken from one of the heifers half an hour after injection, in which case the serum inhibited the growth of the test organism when diluted 1:2 in litmus milk. No penicillin was detected in the milk of either animal.

The dose was therefore increased. One group of four cows were each injected with 500,000 units and a second group of four with 1,000,000 units. Blood samples were taken from all eight animals at half-hourly intervals for three hours and then at hourly intervals for a further three hours. The sera were assayed by the method described above. Two animals of each group were milked out immediately before and every hour after injection and the other two immediately before and five hours after injection. Aliquot portions of each milking were tested for penicillin.

The post-injection inhibitory titres of the blood sera of the eight cows are given as the dilutions of the sera in litmus milk which prevented growth of the test organism. (Table III.) The geometric means of the inhibitory dilutions for the sera of the four cows of each group at each dosage level and at each period following injection were used in constructing the graph shown in Fig. 1. It will be

FIG. 1.—CONCENTRATIONS OF PENICILLIN IN SERUM


No. of tubes in which no change of colour occurred and calculated units of penicillin per ml. of serum.
3
(0·64 1·28 units /ml.)
2
(0·32 - 0·64 units / ml.)
1
(0·16 - 0·32 units / ml.)
½ 1 1½ 2 2½ 3 3½ 4 4½ 5 5½
Time in hours.

×—× Concentration following intramuscular injection of 5×10^5 Oxford units.
O—O Concentration following intramuscular injection of 1×10^6 Oxford units.

seen that with both doses the highest level of penicillin was attained half an hour after injection, and that this level (1/8) was not much greater in animals which received 1,000,000 units than in those injected with 500,000 units (1/6·8). In contrast to this, however, the serum of the cows given the larger dose was inhibitory to the test organism for twice as long (six hours) as that of the animals given

TABLE III

THE INHIBITORY POWER OF SERUM AS DILUTIONS AFTER INTRAMUSCULAR INJECTION OF PENICILLIN

Time of bleeding in hours after injection	*Dose* 500,000 *Oxford units*					*Dose* 1,000,000 *Oxford units*				
	Cow 1	*Cow* 2	*Cow* 3	*Cow* 4	*Geometric mean*	*Cow* 1	*Cow* 2	*Cow* 3	*Cow* 4	*Geometric mean*
½	1/4	1/8	1/8	1/8	1/6·8	1/16	1/8	1/8	1/4	1/8·0
1	1/4	1/4	1/8	1/4	1/4·8	1/16	1/4	1/8	1/4	1/6·8
1½	1/2	1/8	1/8	1/4	1/4·8	1/8	1/4	1/8	1/4	1/5·6
2	1/2	1/2	1/2	1/2	1/2·0	1/8	1/4	1/4	1/4	1/4·8
2½	1/2	1/2	1/2	1/2	1/2·0	1/4	1/4	1/4	1/4	1/4·0
3	<1/2	1/2	1/2	<1/2	1/1·4	1/4	1/2	1/4	1/4	1/3·4
4	<1/2	<1/2	<1/2	<1/2		1/2	1/2	1/2	1/4	1/2·4
5						1/2	1/2	1/2	1/2	1/2·0
6						<1/2	<1/2	<1/2	<1/2	

the smaller (three hours). Selbie *et al.* (1945) found the opposite to hold for man ; larger doses producing higher blood levels without appreciably prolonging the period for which the drug could be detected.

No evidence of the presence of penicillin in the milk of any of the animals was found with either dose.

DISCUSSION

The sensitivity to penicillin of the pathogenic cocci associated with diseases of the bovine mammary gland indicates the possible usefulness of the agent in the treatment of such diseases. Since, however, administration by the intramuscular route of doses as high as one million Oxford units did not result in the excretion in the milk of detectable quantities of the drug, it is improbable that this method of introducing the drug will be of value. The absence of penicillin in the milk when the drug was present in relatively high concentrations in the serum must have been due either to some selective activity on the part of the secretory epithelium of the mammary gland or to the destruction of penicillin by the gland.

The inhibitory effect of bovine serum on the action of bacteriostatic drugs is not peculiar to penicillin as sulphanilamide is similarly influenced. There is, however, a difference between the two drugs, for heating the serum at 56° C. for 30 minutes destroys almost completely the anti-penicillin activity whereas such treatment does not affect the anti-sulphanilamide activity. Thus it appears that some labile factor or factors present in bovine serum act specifically against penicillin. That such a factor is very labile is clearly evidenced by its inactivation by mild heat-treatment and also by the fact that serum kept overnight tends to lose its anti-penicillin activity. If this anti-penicillin action of fresh serum occurs *in vivo* as well as *in vitro*, the fairly high concentration of the drug found in the bovine serum in the present work would have little therapeutic value. Further work is required to investigate this feature.

SUMMARY

Fifty-six strains of *Str. agalactiae* and four of *Staph. aureus* from cases of bovine mastitis occurring in widely separated farms were found to have the same order of sensitivity to penicillin as the Oxford Staphylococcus.

Litmus milk medium is a suitable base in which to estimate penicillin. The results are accurate and easily read.

If fresh serum is present it decreases the inhibitory action of penicillin. This effect, which is directly proportional to the serum concentration, provided that it does not exceed 12 per cent., is due to a particular factor which is thermolabile and is destroyed by heating the serum to 56° C. for 30 minutes. It is not identical with the factor or factors in serum having a similar effect on sulphanilamide.

Such heating does not affect penicillin present in the serum and any serum to be assayed for its penicillin content should be so heated.

Intramuscular injection of cows with doses of 500,000 and 1,000,000 Oxford units gives approximately similar values for the content of penicillin in the serum 30 minutes after injection. With the larger dose, however, penicillin is present in the serum for twice as long as with the smaller dose.

When doses of up to 1,000,000 Oxford units of penicillin are injected intramuscularly, no penicillin can be detected in the milk.

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TRIALS WITH PHENOTHIAZINE-SALT MIXTURES FOR THE PREVENTION OF PARASITIC GASTRITIS IN LAMBS

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INTRODUCTION

Within recent years workers in America have claimed that phenothiazine mixed with ground salt has a marked anthelmintic effect on sheep which are allowed free access to the mixture. The method is said to be of particular value where large numbers of sheep are kept on range and the labour of dosing is very costly.

Thus, in the lamb-feeding areas in California, Britton *et al.* (1942, 1943) using a 1 : 15 phenothiazine-salt mixture, claimed that the daily intake of relatively small amounts of phenothiazine was effective in controlling the common nematodes of sheep and that the mixture was non-toxic when fed continuously for eleven months. The phenothiazine intake in the various trials described varied from 0·28 g. to 0·5 g. per head per day. In one controlled experiment lambs having access to the phenothiazine-salt mixture gained 13·1 lb. more than a similar group getting salt alone. The method was not found to be effective in cattle.

Shorb and Habermann (1940) showed that daily doses of 0·25 to 0·5 g. of phenothiazine given for several days resulted in inhibition of larval development in the faeces of lambs. Habermann and Shorb (1942) also showed that sheep readily took phenothiazine mixtures of from 1 : 9 to 1 : 14, which provided enough of the drug to arrest the development of parasitic larvae in the faeces. The average daily consumption of phenothiazine was at least 0·5 g. per head. In these experiments, although the development of larvae was inhibited, the egg count was not reduced. Thorp and Keith (1943), however, using a 1 : 9 phenothiazine-salt mixture, found faecal egg counts definitely reduced, but they were dealing, in the main, with *Haemonchus* infestations; they state that control lambs died from parasitic gastritis but no weight records were made.

Peterson *et al.* (1944) showed that a 1 : 14 phenothiazine-salt mixture protected sheep from excessive parasitism when they were relatively free from worms at the beginning of the trial and when placed on reasonably clean pasture. The same mixture did not, however, protect sheep when placed on a pasture recently heavily contaminated by parasitised untreated sheep.

More recently, Martin (1944) claimed that scouring was significantly reduced in lambs having access to phenothiazine-salt (1 : 15) mixtures, and that the numbers of *Ostertagia*, *Trichostrongylus* spp. and nodular worms were also reduced in comparison with controls getting pure salt only. The level of parasitism in these experiments was, however, extremely low.

Although the American results, on the whole, have been favourable, workers in Australia have not reported much success. Gordon (1942)

reported the results of some preliminary trials and concluded that the general use of such licks could not be recommended at present because the amounts consumed by sheep are irregular and because little effect was noted on *Trichostrongylus* spp. Moreover, the use of phenothiazine in this way was uneconomical, since better results could be obtained by giving the same quantity as a drench.

Trials in S.E. Scotland, 1943–1945

Sheep management under modern conditions is often a laborious business involving a variety of inoculations, dosings and dippings. If possible, it is very desirable to lighten the load of the shepherd and to cut down the amount of handling of lambs, particularly during the blowfly season. The trials to be described represent attempts to determine whether the method of allowing sheep free access to phenothiazine-salt mixtures would have a useful effect in preventing development of clinical parasitism, under conditions obtaining in this country. It was thought that while this method could not be expected to replace individual dosing of lambs, it might prove a useful supplementary preventive measure, by helping to reduce pasture contamination to a minimum and thus allowing of a less rigorous dosing programme with a consequent saving of labour and handling.

It is important to realise that the method of allowing sheep minute daily doses of phenothiazine aims at cutting down pasture infestation by reducing egg output and inhibiting larval development. It seems unlikely that such small doses have any lethal effect on the worms already present in the sheep.

Preliminary trials were carried out on groups of weaned hill lambs in the autumn of 1943 (Spittal Farm). The main experiments were made on cross lambs running with their mothers on first and second year pastures during the summers of 1944 and 1945 (Saughland).

General methods

In these trials lambs were individually marked, and weighed at intervals. At each weighing, individual faeces samples were taken from as many of the lambs as possible and worm egg counts were made by a modification of the McMaster method. In recording egg counts, *Strongyloides papillosus* and *Nematodirus* spp. were noted separately, the other eggs being included under the head " other species." Where egg counts are referred to in the text they apply to the counts of " other species " unless otherwise stated. No opportunity occurred to make *post-mortem* examinations of any lambs used in the trials, nor were faecal cultures made, so that it is not possible to give an accurate indication of the types of worm present. It may be said, however, that in this region *Haemonchus* infestations are rarely troublesome, but that mixed infestations of *Ostertagia* and *Trichostrongylus* spp. frequently cause losses. In those groups

which were dosed, the phenothiazine was given on the same day as the weighing. This was done for convenience but it meant that in most cases the egg counts taken after dosing were not estimated until four weeks later, by which time they were again rising. Dosing in all cases was done by means of a phenothiazine drench administered with a syringe.

The phenothiazine-salt mixture used in these trials consisted of one part by weight phenothiazine powder (incorporating a wetting-agent as supplied for the drench) and 14 parts of rough ground salt. In the later stages of the 1945 trial this mixture was altered to 1 : 9. Both mixtures were taken well. The mixtures were placed in covered boxes in the appropriate plots ; in all other plots rough ground salt was available. It was found that at first the sheep needed encouragement to go to the boxes to take the mixture. The most successful method was to feed a small quantity of bruised oats for a few days and then, when the sheep were coming readily to the trough, salt was substituted. Phenothiazine was gradually introduced until the full 1 : 14 mixture was in use. During the course of the experiment the boxes had to be filled every five to seven days and occasionally, in rough weather, needed to be cleaned out and refilled with dry mixture.

In the semi-arable sheep farms which are typical of this part of Scotland it is customary to box feed the ewes for some weeks after lambing and this is continued until " the grass comes," about the beginning of June. If the covered boxes are placed close to the ordinary feeding troughs, and the method described above is used, no difficulty is experienced in training the ewes to take the mixture. In due course the lambs follow suit and there is a fairly steady intake by both ewes and lambs throughout the season. Slight discoloration of the fleece down the necks of the lambs was usually noted after a prolonged period on the licks.

It seemed essential to offer the phenothiazine mixture to the sheep early in the season—*i.e.*, before the worm infestation had much chance to establish itself and before the grass was well established. In some small subsidiary trials on other farms difficulty was experienced in getting the sheep to take the lick because the material was introduced too late. During the 1944 season some trials were made on other farms with solid phenothiazine-salt licks prepared for experimental purposes by Imperial Chemical Industries, Ltd. These were not, however, readily taken by the sheep and the trials were abandoned.

The method used to determine the population of infective larvae on the grass was mainly based on the technique described by Taylor (1939).

The grass samples were usually collected early in the morning before the dew had disappeared and were gathered from between 200 and 300 places while crossing and recrossing the pasture in zigzag fashion. A sample weighing 2 to 3 lb., taken from each plot,

was thoroughly mixed in the laboratory and from it a smaller sample, usually weighing 4 oz. (but in some cases, as a check, 8 or 16 oz.) was examined. For the 8 or 16 oz. samples, two or four funnels were used respectively.

The method differed from Taylor's technique in the use of a 0·1 per cent. solution of caustic soda instead of water in the Baermann apparatus. Earlier experiments with this solution showed that larvae were extracted more rapidly than when water alone was used; in fact most of the larvae were separated in two days. Apart from the greater rapidity of extraction the method had the additional advantage that the solution killed nearly all the free-living nematodes, while the infective larvae remained alive. The counting, which was done on a thin streak of suspension pipetted along a slide, under the 2/3 objective of the microscope, was thus facilitated.

In preliminary trials with this method, standard numbers of living *Trichostrongylus* spp. larvae were added in order to form some estimate of the extraction rate, as suggested by Taylor (1939). As no definite advantage could be detected, and, moreover, it was thought that the extraction rate of laboratory cultured larvae could hardly be used as an index of the extraction rate of the mixed population obtained in the field, this part of the technique was abandoned.

The method used was considered suitable for giving a comparison of numbers of larvae present on the three plots at a given time.

DETAILS OF EXPERIMENTS AND RESULTS

Spittal Farm (1943)

This farm is situated on the Pentland Hills at an altitude of 1,000 ft. and was the site of the preliminary trial during the autumn and early winter of 1943. Forty-eight weaned, blackface lambs recently brought from hill pasture and running for about a fortnight on aftermath, were selected at random and distributed in four lots of 12 on four experimental plots each of 3¾ acres. These four plots had all been sown out in 1941, and although their original treatment had differed, the pasture had evened out and there was little to choose between them when the trials commenced. It was known, however, that cattle had grazed Plots 1, 2 and 3 during the summer, but that no cattle had been put on Plot 4. This fact is of interest in view of the results of pasture larval estimations.

There was some suspicion of mineral deficiency on this farm and on one plot a proprietary compound mineral mixture was available to the sheep. This mixture had no apparent effect but the plot provides an additional control to the phenothiazine plots and so will be included here.

Treatment on the plots was as follows :—

Plot 1. Salt and mineral mixture available.
Plot 2. 1 : 14 phenothiazine-salt available.
Plot 3. Salt only available.

Plot 4. Salt available. Lambs dosed 8.11.43 and 29.11.43 with 20 g. phenothiazine.

On all plots the lambs were receiving supplementary feeding consisting of ½ lb. of a mixture of three parts bruised oats with one part linseed cake. The phenothiazine-salt mixture was, on the whole, well taken though the intake varied from time to time with a daily average of about 0·25 g. phenothiazine per lamb.

Results

The results are summarised in Table I. As this experiment was a preliminary trial in which it was not possible to have groups larger than 12 lambs, statistical interpretation is difficult, but if the lame

TABLE I

SPITTAL FARM, 1943. WEIGHTS AND EGG COUNTS

Plot	*Treatment*	*Mean gain in weight*	*Mean egg counts e.p.g.*			
			11.10	8.11	29.11	20.12
		lb.				
1.	Salt + minerals ...	6·6	978	890	862	942
2.	Phenothiazine salt ...	7·3	1,033	577	480	485
3.	Salt only	7·5	1,023	733	890	966
4.	Phenothiazine dosed	9·1 (9·9)*	1,088	768	183	150

* In this group one lamb had a marked hip lameness and lost weight. If this lamb is omitted the average weight gain is 9·9.

lamb from Plot 4 is omitted the mean weight gain (9·9) is just significantly superior to that of Plot 2 (7·3) at the 1 in 20 probability level.

Comparison of logarithms of egg counts suggests that the lower counts in Plot 2 (phenothiazine-salt) show a genuine superiority over those in Plots 1 and 3. It seems permissible, therefore, to draw the following conclusions :—

1. Under the conditions of the experiment dosing with phenothiazine resulted in a slight but significantly greater gain in weight than the phenothiazine-salt treatment, which itself did not show any superiority over the controls.

2. The feeding of phenothiazine-salt mixture produced a drop in worm egg counts which was maintained to the end of the experiment but which was not so marked as that produced by dosing.

The results of the pasture larval estimations shown in Table II

TABLE II

SPITTAL FARM, 1943. PASTURE LARVAL COUNTS. LARVAE PER LB. OF HERBAGE

Plot	*Treatment*	11.10	8.11	29.11*	20.12
1.	Salt + minerals	216	376	108	360
2.	Phenothiazine salt ...	228	288	200	328
3.	Salt only	168	—†	172	236
4.	Phenothiazine dosed ...	408	800	168	480

* Snow on ground.

† Sample accidentally destroyed.

indicate that contamination of pasture was of low grade and there seems to be no difference as a result of treatment of the lambs. It is of interest to note that the initial count on Plot 4 was distinctly higher than those on the other plots and it seems possible that the grazing of cattle on Plots 1, 2 and 3 may have been responsible for the difference.

Saughland (1944–1945)

On this farm a field of approximately 33 acres was divided into three equal plots each supplied with water. In 1944 this field was in young grass which had had a few ewes grazing on it in the early spring. Mr. R. G. Heddle, M.A., B.Sc., Advisory Botanist, Edinburgh and East of Scotland College of Agriculture, reported as follows :—

" The field was visited on June 20th and October 6th, 1944. It is a first year's lea and quite typical of this kind of pasture, the main herbage consisting of Italian and perennial ryegrasses, with cocksfoot well represented and some Timothy. Red and Alsike clovers were present in good amount, and by October white clover had spread to an appreciable extent.

The field was visited again on July 17th, 1945. Taking the field as a whole it is dominated by perennial ryegrass which, at the time of the visit, was fully shot and rather unproductive. Cocksfoot was well represented and in leafy condition. Timothy was present but weak. Some red and Alsike clover survived but the main clover was of the white variety. All plots showed a certain amount of invasion of Yorkshire fog."

Mr. Heddle reported that in both years Plot 1 was distinctly inferior in character to the other two plots—a clearly marked difference which followed the line of the fence between Plots 1 and 2. This was distinctly unfortunate since it placed the lambs on Plot 1 at a great disadvantage. There was little variation between Plots 2 and 3. A chemical analysis of the soil of the plots did not show any difference sufficient to account for the marked inferiority of Plot 1.

1944 Season. In the 1944 season, 18 half-breed ewes with 36 twin lambs (Oxford and Suffolk cross) were put on to each of the plots on April 21st. The ewes in all groups received some box feeding of concentrates up to the end of May.

The groups were dealt with as follows :—

Plot 1. Free access to 1:15 phenothiazine-salt mixture from May 24th.

Plot 2. Lambs dosed monthly with phenothiazine on rising scale of dosage as follows : 20.6.44, 10 g. ; 18.7.44, 15 g. ; 15.8.44, 20 g. All had access to salt.

Plot 3. Controls. Access to salt only.

Lambs were weaned on 12.8.44.

In the 1944 season the weaned lambs were maintained on the same plots and the place of the ewes was taken by an equivalent number of lambs dosed prior to their going on to the plots.

After the 1944 experiment was concluded in late September the lambs were removed and the fences opened. The field was thereafter lightly stocked until mid-November and then left empty until used for a few hoggs in early spring. (The field was at 800 feet and would not provide any winter grazing.)

1945 Season. Since the main object of the experiment was to determine whether the phenothiazine-salt group would show an advantage over the controls, it was decided, in view of the inferiority of Plot 1 (which should be borne in mind when weight gains are considered), to change the layout for the 1945 season. On April 25th the plots were each stocked with 18 half-bred gimmers with 36 twin lambs (Oxford cross) in groups which were arranged as follows :—

Plot 1. Lambs dosed monthly with phenothiazine as follows: 24.5.45, 10 g.; 19.6.45, 15 g.; 17.7.45, 20 g. Access to salt.

Plot 2. Free access to 1:15 phenothiazine-salt mixture from May 4th to June 25th, when the mixture was strengthened to 1:9.

Plot 3. Controls. Access to salt only.

The ewes in all three plots received some box feeding of concentrates up to the end of May.

When the lambs were weaned on July 31st they were removed from the plots and the experiment was wound up with a final weighing on August 13th.

In both seasons the average daily intake of phenothiazine was a little over 0·25 g. per head.

Results

To avoid confusion with the change of grouping the results of the two seasons will be considered separately.

Season 1944. Faecal samples were taken from the ewes as they were put into the plots at the beginning of the trial. The worm egg counts averaged 383, 355 and 506 eggs per g. on Plots 1, 2 and 3 respectively.

The weight records and average egg counts of the lambs are shown in Table III. It will be observed that the control lambs made a satisfactory gain in weight and that there was no advantage

TABLE III

SAUGHLAND, 1944. WEIGHT GAINS AND EGG COUNTS

Plot	*Treatment*	*Mean weight gain*	*Mean weight gain*	*Mean egg counts e.p.g.*			
		23.5 to 15.8	15.8 to 26.9	20.6	18.7	15.8	26.9
		lb.	lb.				
1.	Phenothiazine salt	38·2	6·7	155	225	438	720
2.	Phenothiazine dosed	39·1	6·9	299	175	263	444
3.	Controls	41·4	4·4	276	199	465	970

in favour of either of the phenothiazine groups. The phenothiazine-salt group lambs were at a disadvantage in view of the inferiority of the pasture on Plot 1. The egg counts remained very low until August 15th, but during the following six weeks there was some increase and on September 26th six of the controls were showing counts of over 2,000 e.p.g. It is of interest, therefore, to compare the weight gains during this final period from August 15th to September 26th: these show a definite superiority for both phenothiazine groups over the controls and the differences were found to be statistically significant. The pasture larval counts (shown in Table IV) do not show any difference between the various plots but they are of interest in indicating the gradual build-up of infestation on young grass.

TABLE IV

SAUGHLAND, 1944. PASTURE LARVAL COUNTS. LARVAE PER LB. OF HERBAGE

Plot	*Treatment*	21.4	22.5	20.6	18.7	15.8	26.9
1.	Phenothiazine salt ...	—	28	60	261	591	368
2.	Phenothiazine dosed ...	8	28	68	168	327	248
3.	Controls	44	44	56	80	246	444

Season 1945. The weight gains and egg counts are shown in Table V and the pasture larval estimations in Table VI. From these

TABLE V

SAUGHLAND, 1945. WEIGHT GAINS AND EGG COUNTS

Plot	*Treatment*	*Mean gain in weight*	*Variance**	*Mean egg counts, e.p.g.*				
		24.5 to 30.7		24.5	19.6	17.7	31.7	13.8
		lb.						
1.	Phenothiazine dosed ...	32·4	18·6	1,320	730	320	39	550
2.	Phenothiazine salt ...	35·9	26·1	505	516	346	380	700
3.	Controls ...	28·5	9·6	1,140	960	1,010	770	1,200

Phenothiazine-salt treatment commenced 4.5.45. Phenothiazine dosing (Plot 1) on 24.5, 19.6 and 17.7.

The gains in weight are shown to 30.7.45, when the lambs were weaned and taken off the plots. The post-weaning period to 13.8.45 is illustrated in Fig. 1 (weights).

* The standard errors for differences of means between Plots 1 and 3 and Plots 2 and 3 are 0·89 and 1·00 respectively.

TABLE VI

SAUGHLAND, 1945. PASTURE LARVAL COUNTS. LARVAE PER 1 LB. HERBAGE

Plot	*Treatment*	24.5	19.6	17.7
1.	Phenothiazine dosed	176	340	420
2.	Phenothiazine salt	128	582	436
3.	Controls	152	1,612	1,282

it will be seen that at weaning the lambs on Plot 2 (phenothiazine-salt) showed a superiority of 7·4 lb. in average weight gain over the controls on Plot 3. This difference is more than seven times the standard error (1·00) and must be regarded as highly significant. The phenothiazine-dosed group (Plot 1) showed a superiority of 3·9 lb. over the controls and this was also significant (S.E. = 0·89). It will be remembered, however, that the lambs on Plot 1 were at a considerable disadvantage on account of the inferiority of the pasture and it would not therefore be proper to attach any significance to the difference between the lambs on Plot 2, which received phenothiazine in a salt lick, and those on Plot 1, which were dosed with phenothiazine.

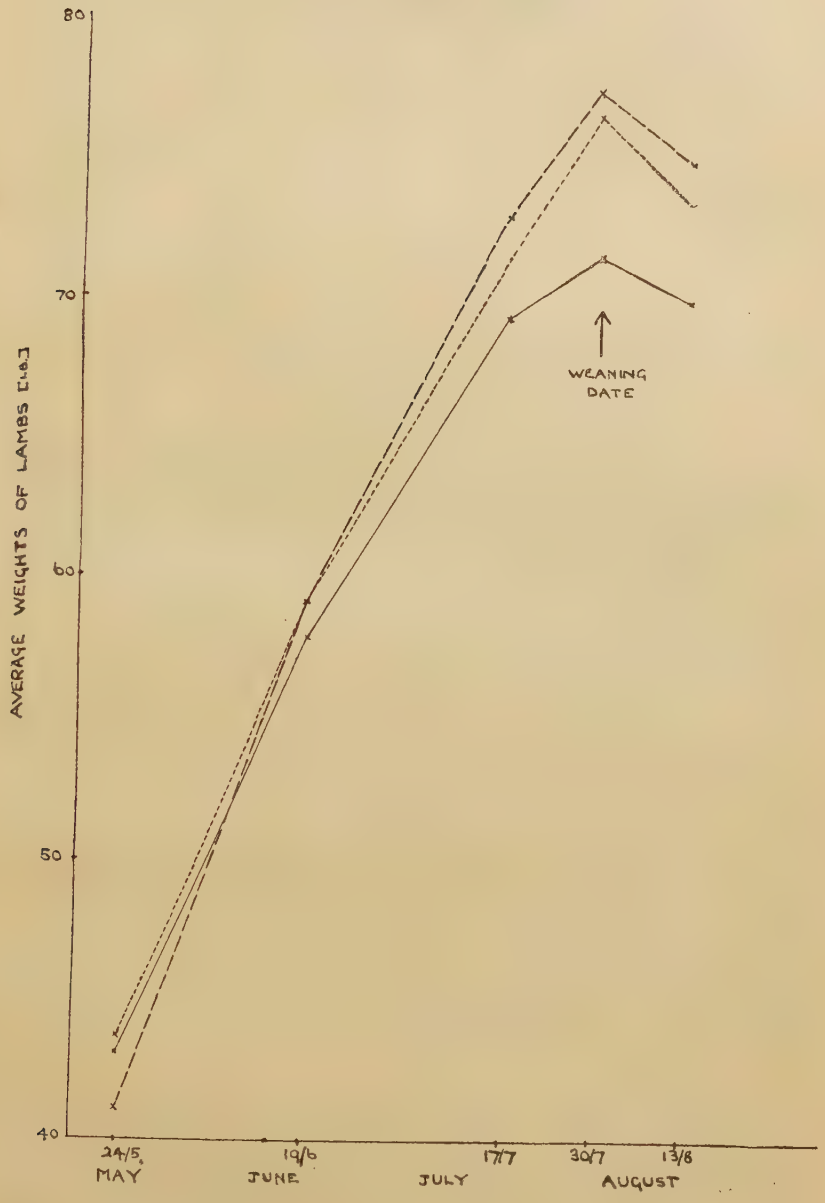
There was an obvious difference in clinical condition between the phenothiazine-salt lambs on Plot 2 and the controls on Plot 3. Most of the control lambs were scouring intermittently during the course of the trial but scouring was practically absent in the group on Plot 2. After weaning the lambs on Plot 3 were obviously " off the thrive " and egg counts rose sharply at this period. Fig. 1 illustrates the difference in weight gain in the three groups and shows the marked check suffered by all three groups after weaning.

Considering the worm egg counts, we find that on May 24th the lambs on Plots 1 and 3 showed counts averaging over 1,000, but those on Plot 2 only 505. The sheep on Plot 2 had already had access to the phenothiazine-salt mixture for a period of three weeks and a good proportion of the lambs were already visiting the trough regularly. It is thought that the mixture had already taken effect and that by the time these samples were examined the egg production on Plot 2 had, probably, dropped to less than half. It will be seen that on this plot the counts remained at a low level throughout whereas on the control plot they remained at a much higher level. On Plot 1 there is an obvious reduction in worm egg count following dosing and this is most marked in the count taken on July 30th, a fortnight after dosing with 20 g. of phenothiazine.

In the method used counts were made in three cells on the McMaster slide and the total number of eggs was noted as the " index " of the worm egg count. For convenience the statistical examination was based on a comparison of these indices for all counts made. Table VII shows the means of these indices, together with the values for n (= number in sample) and s^2 (= mean square or estimate of variance).

If this table is studied it will be seen that both phenothiazine treatments produced a highly significant reduction in worm counts. For example, if comparison is made between the counts on Plots 2 and 3 on July 17th, we find that the variance of the difference between the average indices is 5·81, and the standard error is therefore 2·41. The actual difference between the means (15.2 to 5·2) is more than four times the S.E.

FIG. 1.—SAUGHLAND 1945.
Average weights of lambs.



Key :—

Plot 1. Phenothiazine dosed .....

Plot 2. Phenothiazine/salt - - - - - - - -

Plot 3. Controls ————

TABLE VII
INDEX OF WORM EGG COUNTS

Plot	*Treatment*		24.5	19.6	17.7	31.7
1.	Phenothiazine dosed	M	20·4	11·0	5·3	0·6
		n	22	28	30	31
		s^2	83	97	33	3
2.	Phenothiazine-salt	M	7·6	7·7	5·2	5·7
		n	31	31	27	30
		s^2	69	72	29	28
3.	Controls	M	17·1	14·4	15·2	11·6
		n	27	25	30	30
		s^2	71	56	142	60

M = Mean of worm egg count indices.
n = Number in sample.
s^2 = Mean square or estimate of variance.

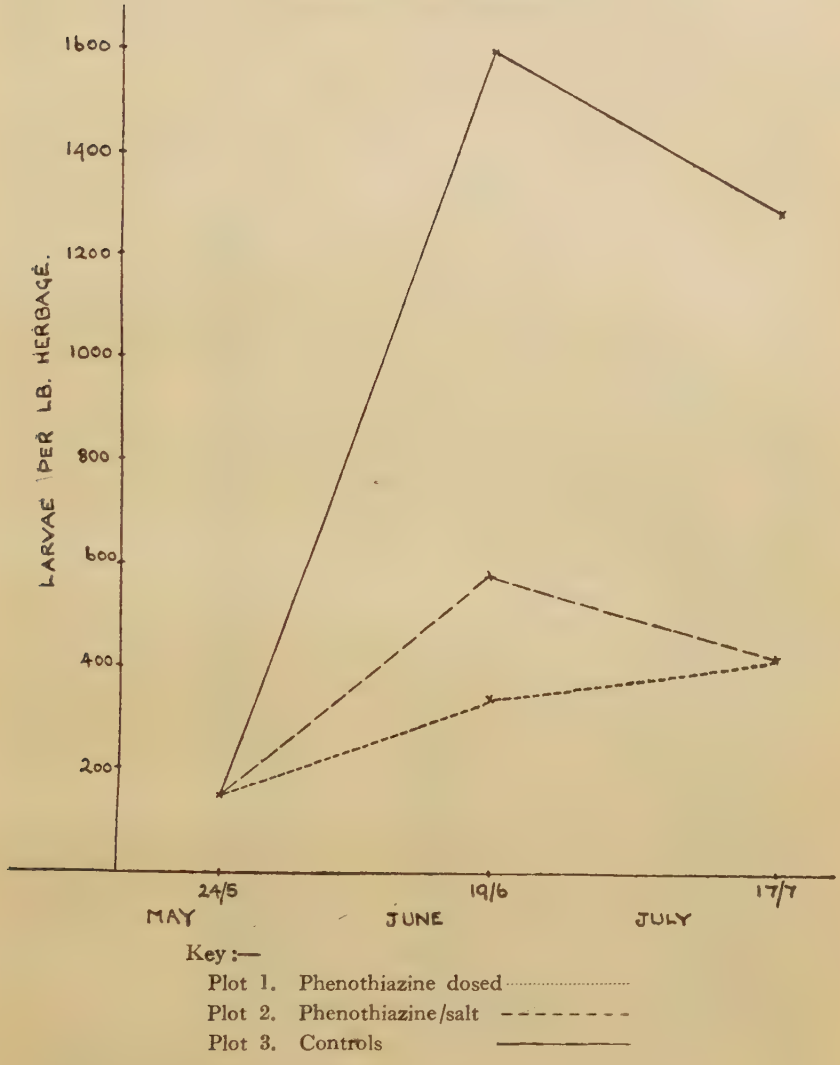
The extraordinarily low mean and practically complete absence of variation in the samples taken on Plot 1 on July 31st can easily be explained as these samples were taken only a fortnight after dosing with maximal doses of phenothiazine (*i.e.*, before there was time for any fresh infestation to show itself in the egg count). The other samples on Plot 1 were taken after intervals of a month from dosing and all show somewhat larger counts and greater variation.

Tests of significance of the pasture larval counts are not possible but it is clear that there was a marked increase in the contamination on Plot 3 as compared with the counts on the two phenothiazine plots which remained more or less at the same levels. These results are illustrated in Fig. 2. The values obtained on Plot 3 are rather higher than those recorded by Taylor (1939), but no great claim to accuracy can be made in regard to *absolute* counts with this technique. It is, however, considered that the method, involving the comparison of numbers of larvae recovered from samples taken by the same sampler on the same day does give a useful comparison of the state of affairs on the three plots. It will be seen that the results fit in well with the egg counts and suggest that with both types of phenothiazine treatment there was a substantial reduction in pasture contamination.

Nematodirus Counts

In counting the worm eggs the figures for Nematodirus spp. were kept separately. The initial counts averaged 500 e.p.g. in the control lambs and these counts showed a progressive fall through the season—the final count being 94 e.p.g. As exactly similar counts were recorded in the other groups it could not be claimed that phenothiazine had any effect on the counts of eggs of these species. Eggs of *Strongyloides papillosus* were present in the young lambs but were never seen in large numbers. In later counts these faded out of the picture altogether.

FIG. 2.—SAUGHLAND 1945.
Pasture Larval Estimations.



DISCUSSION

In the Spittal experiment the results indicated that the use of the phenothiazine licks might have had some effect in lowering egg counts, thus reducing pasture contamination. It may be noted, however, that the lambs were receiving concentrate feeding, stocking was not heavy and at that time of year no marked build-up of infestation could be expected. Phenothiazine dosing produced a slightly greater weight gain but the difference was not sufficient to suggest that under these conditions dosing is an economical proposition.

In the 1944 Saughland experiment the young grass pastures were, at the outset, very clean and the build-up of infestation proceeded only very slowly until August and September when the worm egg counts in the control lambs began to rise. It was clearly seen that dosing of any kind was quite unnecessary before weaning. It is, in fact, the common experience in this part of Scotland that, provided a field is not overstocked and that it has not been overgrazed in the early spring, it is not necessary to dose lambs running with their mothers on young grass fields until after they have been weaned. In the 1944 experiment, it is noteworthy that, although the weight gains in the overall period showed no difference, both phenothiazine groups showed a significant superiority to the controls during the last six weeks of the trial after weaning, *i.e.*, when worm egg counts were rising. It should be noted, too, in considering these results that the phenothiazine-salt group were on a pasture which was distinctly inferior to the other two plots.

In the 1945 Saughland experiment on second year grass we find a much more marked effect (see Fig. 3). Here the pasture infestation was at a higher level and the initial worm egg counts in the control group averaged 1,140 e.p.g. with 63 per cent. of samples showing counts of over 1,000 e.p.g. It is possible that with heavier stocking or in the event of less favourable weather conditions an outbreak of parasitic gastritis might have been produced even before weaning. As it was, the control lambs were showing some loss of condition and much scouring when they were finally dosed a fortnight after weaning. The experiment showed that with the use of the phenothiazine-salt mixture lambs throve better and scoured less, and the pasture remained relatively lightly infested.

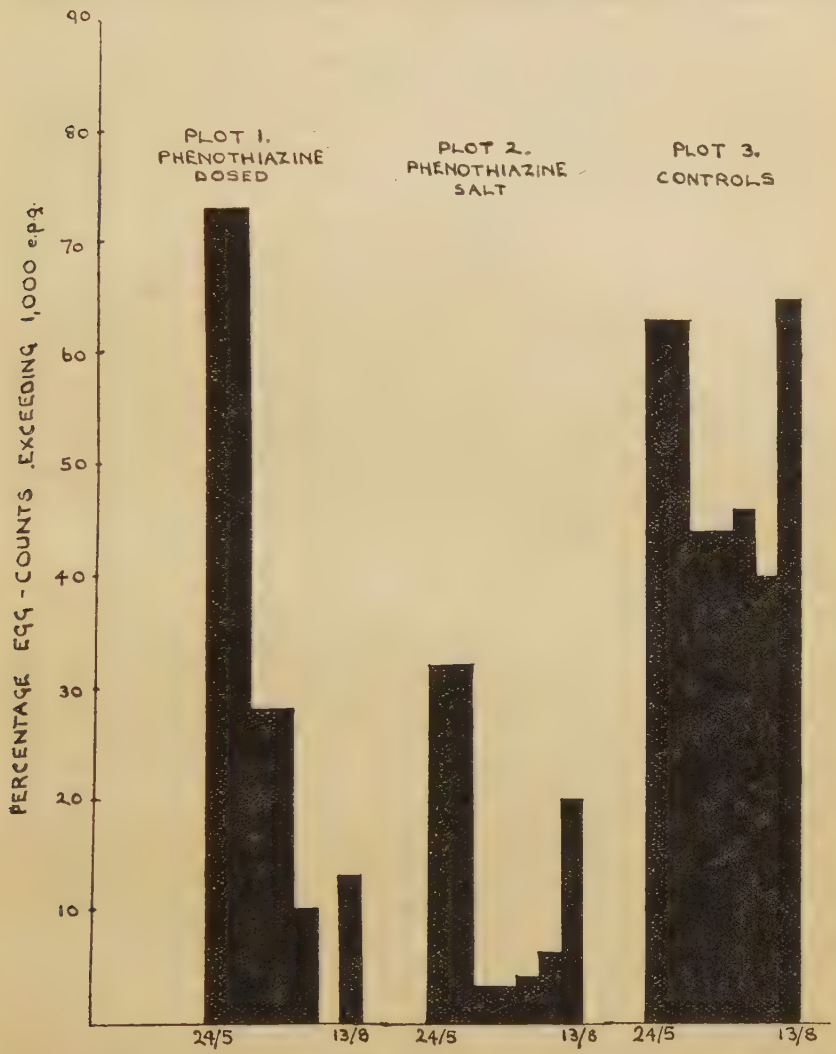
In 1945, worm egg counts were surprisingly high in the early part of the season and some trouble from parasitic gastritis was experienced even prior to weaning on some farms in this area where the stocking was too heavy. Weather conditions may have been partly responsible as the lambs, having got a splendid start with fine open weather during the lambing season, received a check in May during a prolonged period of cold and very wet weather. A spell of fine weather with plenty of sunshine followed in June and July, when lambs generally picked up in condition and made very good progress.

Under the conditions of pasture management and stocking as practised at Saughland, it seemed that the provision of phenothiazine-salt mixtures in boxes was all that was necessary to protect lambs from excessive parasitism during the suckling period and to keep the pasture infestation down to minimal levels. It is considered, however, that further experiments will have to be done before the method could be generally recommended and that such experiments should, if possible, include *post-mortem* examinations, with complete worm counts, of all lambs involved, a procedure which was not possible in our experiments.

Workers in America have described two effects resulting from

FIG. 3.—SAUGHLAND 1945.

Illustrates the effect of allowing access to phenothiazine-salt mixture. The diagrams show the percentage of egg counts exceeding 1,000 e.p.g. in each group.



the daily intake of small doses of phenothiazine (*a*) the inhibition of larval development in the faeces, and (*b*) a reduction in worm egg counts. In our experiments there was a definite reduction in egg counts and this was maintained so long as the lambs were receiving the mixture. When lambs were weaned and taken off the mixture the worm egg counts rose sharply. The question of inhibition of larval development in the faeces was not examined in our experiment.

The daily intake of phenothiazine in all three trials was about 0·25 g. per head. Although sheep took somewhat less of the 1 : 9 mixture of phenothiazine which was instituted during the course of

the 1945 trial, the intake of phenothiazine was approximately the same. Whilst in all trials the intake of plain salt was greater than that of the phenothiazine-salt mixture, even the 1 : 9 mixture was quite palatable.

In view of the success of the method as applied at Saughland, it is perhaps worth while discussing the possible application on a more general scale. It is thought that the method may well prove useful on some farms provided that the farmers using it realise its limitations and are prepared to supplement its effects by judicious dosing. A possible scheme might be as follows :—

1. Dose the ewes six weeks after lambing and introduce phenothiazine-salt mixtures on all pastures where sheep are running.
2. Keep a watchful eye on the lambs ; any individual lambs showing signs of scouring or loss of condition could be dosed in the ordinary way.
3. After weaning dose all lambs with 20 g. phenothiazine and maintain phenothiazine-salt mixtures on all pastures.
4. In the second year introduce phenothiazine-salt mixtures to the ewes during the last weeks of pregnancy and dispense with the dosing of ewes.

This scheme is suggested on a basis of the American and of our own results, but it is emphasised that it should be applied in conjunction with good management of grazing in order to minimise pasture infestation and is not to be regarded as anything but preventive in character. It is not considered that the method is likely to succeed on second and third year pastures which have been very heavily stocked in the previous autumn and winter, but if a start is made with a moderately clean pasture the use of phenothiazine-salt mixtures should reduce the infestation on the grass to the level at which a balance is set up between host and parasite and the latter does not increase in numbers to the detriment of the former.

The method is not as efficient as dosing, but where labour is a difficulty and the blowfly is a menace the use of the mixture may be worth while in order to cut down the amount of dosing which has to be done. Where cattle or horses are grazed together with sheep it will be necessary to protect the boxes by means of a wire fence, as is usually done with feeding boxes in the early summer in fields where other animals are grazing.

Cost. The phenothiazine consumed by the lambs taking the mixture amounts to about half of that which would be used in a monthly dosing programme in lambs, but since the ewes also take the mixture the actual consumption of phenothiazine may be very much the same as in a dosing scheme. Thus no claim to economy can be made for this method apart from the question of labour costs.

SUMMARY

Experiments are described in which comparison of weight gains, worm egg counts and pasture larval estimations respectively were

made in groups of lambs (*a*) treated with phenothiazine, (*b*) allowed access to phenothiazine-salt mixtures, (*c*) untreated controls.

The trials were carried out in weaned hill lambs on second year grass in autumn and winter, and in suckling lambs running with their mothers on first year grass (1944) and second year grass (1945).

In all cases it was found that the use of phenothiazine-salt mixtures caused a depression in worm egg counts. In the 1944 season, *i.e.*, on first year grass, the level of parasitic infestation remained very low until the post-weaning period, when the phenothiazine groups gained a slight advantage over the controls. In the 1945 experiment the level of parasitic infestation was sufficient to cause loss in condition and in this trial the lambs on phenothiazine-salt showed a significantly greater weight gain than the controls. The same effect was noticed in the group dosed with phenothiazine. On the control plot the pasture larval counts rose to a high level but those on the two phenothiazine plots remained moderately low.

It is suggested that phenothiazine-salt mixtures might prove useful in keeping pasture infestations at a low level and thus reducing the amount of dosing required, but it is emphasised that further experiments would have to be done before the method could be generally recommended for use in this country.

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STUDIES ON *CORYNEBACTERIUM RENALE*

I.—A SYSTEMATIC STUDY OF A NUMBER OF STRAINS

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INTRODUCTION

A specific cystitis and pyelonephritis of cattle associated with a diphtheroid is recognised on the Continent of Europe, in this country and in America. The passage of turbid or bloodstained urine is a characteristic symptom and diphtheroids are found in the urinary sediment and the pelves of the kidneys. Opinions differ as to the route whereby these bacteria reach the kidneys and bladder, and some confusion exists concerning the identity of the bacteria and their behaviour in the laboratory. Although *Corynebacterium pyogenes* is occasionally involved in a suppurative nephritis of cattle and other animal species, the common organism associated with this specific cystitis and pyelonephritis of cattle is one known as *Corynebacterium renale.*

Enderlen (1891) and Höflich (1891) studied the disease and the associated bacterium, and Bollinger (1891) suggested *Bacillus renalis bovis* as a suitable name for the organism; Ernst (1905) called it *Corynebacterium renalis.* The primary cultures of these early workers were often mixed and the role this organism played in the pathogenesis of the disease in cattle was obscure; experimentally it was usually non-pathogenic for laboratory animals. Ritzenthaler (1910) reviewed the available information and concluded that *C. renale* was of aetiological importance and he stressed its predilection for urine and its power to split urea. Some years later Jones and Little (1925, 1926, 1930) described cases of the disease in America and recorded their observations on the bacteria they recovered. They studied 26 strains from the kidneys or urine of diseased cattle and 128 from the urinary tract of 34 calves in a herd in which cystitis and pyelonephritis were known to exist. The 26 strains from diseased cattle and 25 of the strains from 12 of the 34 calves were similar regarding morphology and cultural characteristics; there was also a serological relationship as tested by the agglutination reaction and by a limited number of agglutinin absorption tests. Their cultures were non-pathogenic for rabbits, guinea-pigs and white mice and a soluble toxin could not be demonstrated. A number of experiments were made with cows; some were injected intravenously with broth cultures and failed to develop the disease, whilst in others the introduction of small quantities of culture into the bladder by way of the urethra gave rise to a cystitis which in some cases was transient. Merchant (1935) included 20 strains of *C. renale* in his study of the corynebacteria associated with diseases of domestic animals. He found that *C. renale* was variable in its ability to ferment carbohydrates and not all of his strains were agglutinated by sera prepared against three strains. Included in his study were twelve diphtheroids which he called *C. pseudotuberculosis* and there is the implication that these are similar to *C. ovis;* none of these strains showed lysis when grown in

rabbits' blood agar and eleven of these were of bovine origin. A serological relationship, however, existed between some of them and some of his strains of *C. renale*. Six of 19 strains examined by Feenstra *et al.* (1945) differed from what they considered to be typical *C. renale* by digesting milk more vigorously and producing acid in xylose.

C. renale is generally accepted as being a large Gram-positive diphtheroid with metachromatic granules and is capable of growth on agar and in broth. Cultures may be white to pale yellow in colour on solid media and a sediment of bacteria occurs in fluid media. Glucose is the sole common carbohydrate which is fermented, and litmus milk becomes alkaline and deep blue in colour with slow digestion of the casein. Most strains show a serological relationship with each other and none appears to be pathogenic for laboratory animals.

Diphtheroids which occur in bovine urine and cannot be classified as *C. renale* are relatively common and even some of those so designated differ from the classical description in their biochemical and serological characteristics. Observations have therefore been made on the behaviour of a number of strains of diphtheroids isolated from animals with an especial attempt to separate typical *C. renale* from others with which this organism may be confused.

METHODS USED AND RESULTS OBTAINED

Most of the diphtheroids studied were isolated in this laboratory during the last few years. The majority of the strains were recovered from the kidneys of cattle which on *post-mortem* examination showed typical changes of pyelonephritis, or from the urine of cattle showing the clinical signs of pyelonephritis.

Ten strains which were isolated from the diseased kidneys or bladder of typical cases of disease are taken as representative of *C. renale*. Of 16 strains isolated from the urine, 15 were from cattle which showed clinical signs of pyelonephritis, and one was from the urine of a cow which appeared normal but was in a herd in which pyelonephritis was known to occur. It will be observed that the greater proportion of those from urine are similar in their reactions to those recovered from the kidneys and bladder. These 26 strains isolated from specimens of kidney or urine were from cattle in the counties of Berkshire, Devon, Essex, Hampshire, Herefordshire, Hertfordshire, Kent, Northamptonshire and Surrey; this information is given to indicate that bovine pyelonephritis is widespread throughout the area from which specimens are received in the laboratory.

In order to effect some form of comparison a few other diphtheroids were included in the study and a brief record is appended of ten strains. These consisted of five cultures comprising one each of *C. pyogenes*, *C. ovis* and *C. equi*, and two which are closely allied to *C. renale*; the latter originated from the prepuce of a bull calf and the kidney of an ox respectively. Of the remainder, two were

from the semen of bulls and show some characteristics common to *C. renale*, and three from the brain of a sheep, milk and udder secretion respectively. A list is appended showing the origin and history of the strains studied. (Table I.)

TABLE I

HISTORY OF STRAINS OF DIPHTHEROIDS STUDIED

Laboratory number	*Site of isolation*	*Designation*
1–10	Kidneys and bladder of cattle showing cystitis and pyelonephritis	*C. renale*
11–26	Urine of cows showing clinical signs of pyelonephritis except No. 25, which was from the urine of a normal cow in a herd in which pyelonephritis was known to exist	*C. renale*
27	Bovine pyaemic nephritis	*C. pyogenes*
28	Received from Australia	*C. ovis*
29	„ „ Sweden	*C. equi*
30	Prepuce of bull calf	? *C. renale*
31	Kidney of cow (N. Ireland)	? „
32	Milk	—
33	Brain of sheep	—
34	Bovine mastitis	—
35	Semen of bull	?
36	„ „	?

MORPHOLOGICAL, CULTURAL, BIOCHEMICAL AND PATHOGENIC CHARACTERISTICS OF *C. RENALE*

Morphology of C. renale

C. renale is a large Gram-positive diphtheroid, and when grown on agar, blood agar, or milk agar for 24 hours at 37° C., the individual cells vary from slender to coarse rods, lying at acute angles or in bundles. There is a marked tendency for them to appear in masses in natural material such as infected urine. The ends of the individual cells are pointed and when stained appropriately metachromatic granules are observed. All of the ten strains from kidneys and all of the 16 from urine showed metachromatic granules. All the miscellaneous diphtheroids were Gram-positive but varied in actual morphology and three strains (30, 31, 32) showed metachromatic granules.

Cultural characteristics of C. renale

Growth occurs readily in broth and on plain agar, reaching its maximum after two to three days at 37° C. A faint surface veil occurs in broth after about 24 hours' incubation and thereafter a deposit of bacteria develops and the supernatant fluid is relatively clear. The colonies on solid medium are dry and granular, varying

in size up to 1·5 mm. in diameter. The colour of the growth is usually cream to pale yellow, increasing in depth after cultures have remained at room temperature for a few days. There is no marked improvement in growth on media to which blood or serum is added. Blood is not haemolysed, serum is not liquefied but the pigment is usually marked on blood agar, solid serum and egg medium; catalase is produced. Of the miscellaneous group *C. pyogenes* and *C. ovis* are haemolytic species and the former liquefies solid serum and egg medium.

A characteristic change occurs on agar to which 10 per cent. of sterilised milk is added. The colonies of *C. renale* develop with cream and pale yellow colour pigment and after 48 hours' growth each colony is surrounded by a halo or zone of clearing (see Fig. 1). There is no advantage in incorporating litmus, bromo-cresol purple, normal rabbit serum or specific immune serum in the milk agar. The haloes become indistinct after 96 hours' growth; the optimum time of incubation is 48 hours and the use of milk agar is of great value for the isolation of *C. renale* from diseased tissue and infected urine. Nine of the ten strains from kidneys showed the clearing zones and 14 of the 16 strains from urine reacted in a similar manner. Two of the miscellaneous strains (31 and 35) also formed haloes. A limited number of tests were made with media containing 10 per cent. milk, 10 per cent. separated milk, 10 per cent. milk which had been treated with ether, 10 per cent. casein and 10 per cent. butter fat. The clearing-zones developed on the media containing milk, ether-treated milk and separated milk, but not on that containing casein or butter fat. The reaction is therefore not a lipolytic one but probably depends on a solution of caseinogen.

Biochemical reactions

A marked reaction occurs when *C. renale* is grown on litmus milk. After two or three days' incubation the medium becomes alkaline and thereafter a granular deposit develops with a clear supernatant fluid the colour of burgundy. The height of the reaction is seldom seen for seven to ten days. Nine of the ten strains from kidneys, 15 of the 16 strains from urine, and four of the miscellaneous strains (30, 31, 35, 36) produced this change.

As the older workers had stressed the ability of *C. renale* to split urea, a medium was prepared consisting of broth to which 1 per cent. urea and 12 per cent. thymol blue (B.D.H.) were added. The medium is pale brown in colour and the production of a dark blue colour occurred with strains of *C. renale* after 24 hours' incubation at 37° C. This was taken as evidence of the breakdown of the urea and the formation of alkali. All of the ten strains from kidneys, 15 of the strains from urine and four of the miscellaneous strains (30, 31, 35, 36) produced this reaction.

Carbohydrate fermentation was tested by growing strains in peptone water containing the requisite substance and Andrade's

reagent. Glucose is the only one, of those tested, which was regularly and consistently fermented and this occurred without the formation of gas. The production of acid was apparent after 48 hours' incubation at 37° C. The carbohydrates tested and the number of strains which fermented them are given in Table II. The methyl red test and the Voges-Proskauer test were made after five days' growth at 37° C. in glucose phosphate medium. The Barritt (1936) modification was adopted for the V.P. test. Eight of ten strains from kidneys

TABLE II

MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL REACTIONS OF THE STRAINS STUDIED

	Strains of C. renale *from kidneys*	*Strains of* C. renale *from urine*	*Miscellaneous strains of diphtheroids*
Number examined ...	10	16	10
Metachromatic granules	10	16	3
Haloes on milk agar	9	14	2
Fermentation of :—			
Glucose	10	16	6
Maltose	0	0	1
Mannite	0	1 (tr.)	0
Lactose	0	0	0
Sucrose	0	0	0
Salicin	0	0	0
M.R.	8	15	1
V.P.	8	16	6
Alkalinity with clearing in litmus milk	9	15	4
Urease activity ...	10	15	4

gave positive M.R. and V.P. tests, and 15 of the strains from urine reacted in a similar manner, whilst one strain from urine was V.P. positive and M.R. negative. One of the miscellaneous strains (30) was M.R. and V.P. positive, and five others (28, 29, 34, 35, 36) were M.R. negative and V.P. positive.

Serological reactions

Immune sera were prepared in rabbits by the intravenous injection of formalinised suspensions of three different strains of *C. renale*, all of which had been recovered from diseased kidneys. They were from specimens received from Hampshire, Hertfordshire and Northamptonshire.

Owing to the ready flocculation of suspensions of *C. renale* in a fluid medium the agglutination test was not used and experiments were made with complement fixation and the precipitin reaction. It was shown that the complement fixation test revealed a close serological relationship between those strains of *C. renale* tested, and a limited relationship existed also between them and certain other

diphtheroids. This test, however, was discarded in favour of a precipitin test using the immune sera and an acid extract similar to that used for grouping streptococci.

The test organism was grown in 30 c.c. of broth at 37° C. for 48 hours and the culture was centrifuged and the deposit washed in saline. The washed deposit was treated with 1 c.c. of N/20 HCl in saline and placed in a bath of boiling water for 15 minutes. It was allowed to cool and was then neutralised with NaOH using phenol red as an indicator. The mixture was again centrifuged and the supernatant fluid mixed with immune serum in capillary tubes, and a positive result was accepted when a clear-cut precipitate developed at the junction of the two fluids within a few minutes at room temperature. A delayed and slight precipitate was apparent with extracts from some other diphtheroids. The three immune sera and the extracts from the corresponding strains reacted strongly with each other and there appeared to be no difference in reaction which serum was used. The results with extracts from the ten strains recovered from kidneys showed that seven reacted strongly whilst 15 of those from the 16 strains recovered from urine reacted similarly and slight precipitates were formed with extracts from five of the miscellaneous strains (28 (*C. ovis*), 30, 31, 35, 36).

Experimental pathogenicity

The records of the pathogenicity of *C. renale* for experimental animals are meagre and generally negative; it was decided to re-investigate the question, using mice as the test animals. Preliminary work included the injection of serum obtained from a rabbit immunised against mouse kidney in the hope that the use of such a serum might induce the localisation of *C. renale* in the kidneys. A series of mice were injected by different routes with living suspensions of *C. renale* and in some cases the suspensions were mixed with the serum of the rabbit immunised against mouse kidney tissue.

The preliminary experiment consisted of injecting by the intraperitoneal route, three mice with kidney serum, six with a living suspension of *C. renale* and six with kidney serum, followed 45 minutes later with *C. renale*. The mice were killed after ten and 25 days, and no *C. renale* were recovered from the tissues. Eleven female mice were given injections of nembutal, six of which were also given kidney serum; when under the influence of the anaesthetic living *C. renale* were injected into the urethra of all of them. No *C. renale* were recovered from the tissues of the mice killed up to 28 days later. Twelve mice, half of which had been given anti-kidney serum, were injected intravenously with a suspension of *C. renale*. Five of six receiving serum and suspension died from seven to 28 days later and *C. renale* was recovered from the kidneys. Two of the six receiving *C. renale* alone were found with *C. renale* in the urinary organs, one died after ten days and the organism was recovered from the kidneys, and the other after 32 days, with *C. renale* in the urine.

A further experiment was made using one strain of *C. renale* in a dose of approximately 20 million living organisms. Mice were injected intravenously and killed at intervals from seven to 21 days later. *C. renale* was recovered from the kidneys and urinary tract of three of 16 mice given a living suspension of the organism and from six of 15 mice previously given an injection of anti-kidney serum. Anti-kidney serum alone was injected into eight mice and these mice were killed and were normal when examined *post mortem.*

It appeared as if the intravenous injection of living suspensions of *C. renale* into mice in adequate amounts is followed in a proportion of cases by infection of the kidney and bladder ; the infection appears to take effect from about seven days after injection, and in some cases at least there is obvious enlargement of kidneys with pus in the medulla and bloodstained urine in the bladder. A number of mice may die, whereas some apparently survive the infection.

It was decided to test the pathogenicity of the strains of *C. renale* and other diphtheroids in this way and suspensions were prepared and given intravenously to mice ; cultural examinations were made of the blood, spleen, kidneys and urine of those which died and of the survivors which were killed seven days after injection.

Three mice were used for each of seven strains from kidneys and six for each of the three remaining strains ; for the urine strains, one was injected into six mice and 15 into three mice each ; two of the miscellaneous strains were injected into six mice each and eight into three mice each. All cultures were prepared on milk agar plates and the results are given in Table III.

TABLE III

RESULTS OF THE CULTURAL EXAMINATION OF THE TISSUES OF MICE INJECTED WITH THE STRAINS STUDIED

Number and designation of strains	*Number of mice injected*	*Number from which the injected strain was isolated*			
		Blood	*Spleen*	*Kidneys*	*Urine*
10 (*C. renale* from kidneys)	39	2	2	28	28
16 (*C. renale* from urine)...	51	2	5	36	33
10 (miscellaneous strains of diphtheroids) ...	36	4	6	5	3

It is thus clear that as far as *C. renale* is concerned it was possible to recover the organism from the urinary tract in a high proportion of cases, and although it is not suggested that this is in any way a specific reaction there is a marked distinction obtaining with the ten strains of miscellaneous diphtheroids.

DISCUSSION

There is little doubt that *C. renale* as met in this country is similar in behaviour to that recorded in the European and American literature. It is, however, difficult to escape the conclusion that other

strains of diphtheroids occur which may or may not be classified as *C. renale* depending on the criteria used. According to the findings herewith recorded *C. renale* is a large Gram-positive diphtheroid showing metachromatic granules, capable of growth on plain agar and forming haloes around the individual colonies when grown in 10 per cent. milk agar; it forms acid from glucose but no other common carbohydrate, renders litmus milk alkaline with subsequent clearing of the medium, splits urea and gives positive M.R. and V.P. reactions; a precipitate is formed with immune serum and acid extracts, and when injected intravenously in mice the organism may be recovered from the kidneys and bladder of a high proportion of the mice seven days later. If these criteria are accepted as typical of *C. renale*, then several strains included as *C. renale* should not be named as such because of the lack of one or more characteristics. For example, slight or no precipitate is formed when immune serum is mixed with extracts from strains 5, 8, 10, 24; the following fail to form haloes on milk agar: 5, 19, 24, and numbers 8 and 9 are M.R. and V.P. negative, whilst 24 is M.R. negative but V.P. positive; number 12 does not split urea. Six strains of the 26 from infected kidneys and urine therefore fail to fulfil all the requirements as herewith accepted; it is of course clear that final judgment cannot be passed and it is probable that the lack of certain characteristics will be accepted merely as a variation from the norm.

There is no difficulty in differentiating *C. renale* from those other diphtheroids of animal origin which are well defined. For example, *C. pyogenes* requires an enriched medium for growth, liquefies coagulated serum and egg and produces a toxin which is lytic for red cells; it clots and digests milk after primary acid production, and may give a faint clearing on milk agar. *C. ovis* is well recognised and forms a toxin; it produces lysis of red blood cells and forms acid from glucose and maltose; the strain herewith studied formed alkali with subsequent clot and some decolorisation in litmus milk, grew on milk agar without halo-formation, did not display any urease activity and gave a negative M.R. and a positive V.P. reaction. *C. equi* grows heavily on plain media with a cream moist and often pigmented growth; it ferments no carbohydrates and produces no change in litmus milk. The strain studied herewith showed slight but late urease activity and gave a negative M.R. reaction and a positive V.P.

SUMMARY

C. renale is a large Gram-positive diphtheroid, possessing metachromatic granules. It grows readily in broth and on agar with cream to yellow colonies.

When grown on 10 per cent. milk agar colonies of *C. renale* are surrounded by a halo or zone of clearing. This reaction is of great value when isolating *C. renale* from animal tissue.

C. renale produces a characteristic alkaline change in litmus

milk; splits urea, ferments glucose and gives positive M.R. and V.P. reactions.

Most of the strains of *C. renale* tested appear to be serologically related as judged by the precipitin test.

C. renale is pathogenic for mice, provided an adequate amount is given intravenously and the organism may be isolated from the kidneys and bladder of a high proportion of those which die and those which survive for seven days.

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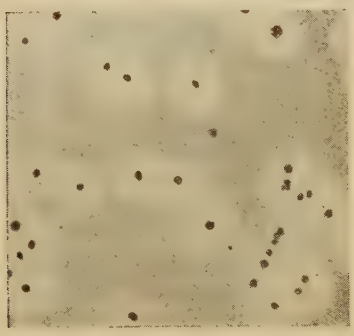

FIG. 1.—Colonies of *C. renale* on 10 per cent. milk agar. (37° C., 48 hours growth). × 3.

STUDIES ON *CORYNEBACTERIUM RENALE*

II.—THE EXPERIMENTAL PATHOGENICITY FOR MICE

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INTRODUCTION

There is a general opinion that *Corynebacterium renale*, although responsible for a pyelonephritis and cystitis in cattle, is non-pathogenic for laboratory animals.

Enderlen (1891) induced lesions of pyelonephritis in two rabbits by the intravenous inoculation of *C. renale* after he had ligatured the ureters, but other methods of infection gave negative results. The cultures of *C. renale* used by Jones and Little (1926) were non-pathogenic for rabbits, guinea-pigs and white mice. They found that rabbits easily withstood an intraperitoneal inoculation of 10 c.c. of a 48-hour broth culture and that guinea-pigs and white mice were equally resistant. There is also some confusion as to the route whereby this organism reaches the kidneys and bladder in natural cases of disease. Enderlen's limited experience with rabbits and Ernst's (1905) observations that in some cases of bovine pyelonephritis the bladder and urethra were not infected, supported the view that the route is a haematogenous one. On the other hand Jones and Little feel certain that the infection is not haematogenous since cows injected intravenously with cultures failed to develop cystitis while small quantities of cultures introduced into the bladder by way of the urethra gave rise to a typical persistent cystitis.

It has been recorded in the previous paper (Lovell, 1946) that *C. renale* is pathogenic for mice provided adequate amounts are given intravenously, as is evident from examinations of kidneys and bladder of those which die and those which survive for seven days or more. This observation suggested further study and accordingly experiments were made involving histological as well as bacteriological examinations. The main enquiry concerned the possibility that *C. renale* may have a selective action on the kidney and bladder of the mouse; on the other hand, the results obtained may be the expression of excretion phenomena. If actual multiplication occurs in the kidney tissue and if a disease similar to that in cattle develops, then further studies in other animal species are called for. It should also be possible to utilise mice for experimental procedure and immunity studies, and these may give indications for profitable work in cattle. Gorer (1940) and Dunn (1944) found renal lesions occurring spontaneously in certain in-bred strains of mice, the necrotic lesions they found in the renal papilla being in some ways not unlike those we have observed after inoculation of *C. renale*. Their mice, however, were much older than the ones we used, and we have not observed the amyloid changes which Dunn found. The lesions which developed in our mice are inflammatory and not degenerative in origin.

METHODS OF STUDY

The strain of *C. renale* used in all the experiments described here had been recovered from the kidney of a cow with typical pyelonephritis and conformed to all the criteria which are accepted as characteristic of *C. renale* (Lovell, 1946). In nearly all cases the inocula consisted of living suspensions which were prepared by washing off the growth obtained on agar slopes, and these suspensions were standardised by an opacity method. The probable numbers of viable organisms were estimated by making a comparative study of the number of viable organisms present in one suspension of a given opacity. Unless stated to the contrary, the approximate number of living bacteria was calculated from the result obtained. The mice were about eight weeks old and weighed from 15 to 20 g. Cultures from those which died or were killed were made on to 10 per cent. milk agar plates. *C. renale* recovered from the experimental mice were identified by observing their morphology and reaction to the Gram stain, their ability to form haloes around the colonies on milk agar, the fermentation of glucose but not maltose and the formation of alkali from urea.

All the experimental mice were given a serial number and as far as possible equal numbers of male and female mice were inoculated. Left kidneys were taken for section and right kidneys for culture, from mice with odd numbers, and the reverse from mice with even numbers. Thus it was possible to minimise any discrepancy due to sex or kidney peculiarity which might otherwise occur. In one experiment there was a suggestion that *C. renale* tended to localise in the kidneys of female mice earlier than in males, but this did not occur in subsequent tests.

Kidneys and portions of other tissues examined were fixed in 10 per cent. formalin or, in some cases, in a fixative described by Cleverdon (1943) and paraffin sections were prepared; these were stained by haematoxylin and eosin and by Gram.

THE SEQUENCE OF EVENTS FOLLOWING INTRAVENOUS INOCULATION OF *C. renale*

The first experiment was designed to give a general picture of the sequence of events which followed the inoculation of *C. renale* intravenously into mice. A total of 50 mice, 25 females and 25 males, were given approximately 20 million of viable organisms intravenously and two mice of each sex were selected at intervals and killed; these were examined bacteriologically and sections of tissues prepared for histological examination. Of the 50 mice injected 14 died; two of these were not suitable for examination. From about the fourth day the surviving mice showed signs of ill-health and the kidneys of those which were killed or died at about the seventh day were frequently large and pale; inspissated pus containing enormous numbers of *C. renale* was sometimes found blocking the pelvis of the organ. The details of the bacteriological

and histological examinations of the 36 mice which were killed are given in Table I, and the details of the 12 which died and were available for examination in Table II.

TABLE I

THE DISTRIBUTION OF *C. renale* IN MICE KILLED AFTER INTRAVENOUS INOCULATION

Number examined	*Interval after inoculation*	*Number showing presence of* C. renale				*Lesions in kidneys*
		Blood	*Spleen*	*Kidneys*	*Urine*	
4	6 hours	4	4	4	0	0
4	24 „	0	1	3	2	0
4	30 „	0	1	4	2	1
4	2 days	0	1	3	3	3
4	3 „	0	0	4	4	3
4	5 „	0	0	3	3	3
4	7 „	0	0	4	4	4
4	10 „	0	0	3	4	3
4	14 „	0	0	2	4	4

Although no enumeration of the organisms recovered was made it was clear that the concentration of *C. renale* increased in the kidneys from about the third day and reached the maximum at about the seventh day; there was a marked reduction by the 14th day. The concentration in the urine occurred later and was at its height at about the tenth to the 14th day. In view of the small size of some of the lesions it is possible that, had serial sectoins of the kidneys been cut, more lesions would have been detected.

TABLE II

THE DISTRIBUTION OF *C. renale* IN MICE WHICH DIED AFTER INTRAVENOUS INOCULATION

Number died	*Day of death*	C. renale *recovered from*			
		Blood	*Spleen*	*Kidneys*	*Urine*
1	3rd	1	1	1	1
3	5th	0	0	3	3
2	6th	0	0	2	2
3	7th	0	0	2	3
1	10th	1	1	1	1
2	12th	0	0	2	2

All of the above mice had severe lesions in the kidneys.

From a study of the kidney sections of the mice of both groups a general picture emerges and the development of the lesions appears to be as follows: Organisms were seen first in clumps and appeared to be in the intertubular capillaries between the collecting tubules near the apex of the papilla. A hyaline eosinophil Gram-negative material was also seen in the same region; the nature of this material was not determined but it may represent a local thrombosis with or without bacterial embolism. A local dilatation of capillaries occurred,

and numerous polymorphonuclear cells accumulated in them which later passed through the normal or necrotic epithelium of the collecting tubules into their lumen.

Small abscesses developed in the papilla, often just beneath the visceral pelvic epithelium, and pus was seen in the renal pelvis as early as 30 hours after the inoculation of the bacteria. Necrosis of the tip of the papilla was an early change (see Fig. 1) and this extended back towards the medulla, involving in so doing the whole (as in Fig. 2) or only part of the width of the papilla. Numerous large clumps of Gram-positive diphtheroids were observed in the necrotic material and a zone of polymorphonuclear cells was present at the extending edge of the necrotic area (see Fig. 3). Streaks of similar cells were also found extending up through the medulla towards, and in some cases into, the cortex; these cells were almost entirely between the tubules, but were occasionally present in their lumen. In four cases these cells were present in between the cortical convoluted tubules, but the local glomeruli were not involved.

The renal pelvis became dilated, often markedly so, and the parietal pelvic epithelium also became inflamed, showing local abscess formation and ulceration. In two cases the inflammation spread to the perirenal tissue (in one mouse that died on the fifth day, and in one mouse killed on the tenth day), while a ureteritis and peri-ureteritis were seen in three cases (in one killed on the seventh day, one killed on the 14th day, and in one dying on the fifth day). One mouse only showed signs of a cystitis (died on the 12th day). On the other hand, pus cells were often seen in the lumen of the bladder.

From this it appears that the earliest and the main lesions developed in the renal papilla and were chiefly a papillitis, pyelitis and pyonephrosis, with a spread of inflammation down the ureter and to the perirenal tissue. None of the mice that died showed cortical lesions, and in those which were killed there was a general absence of cortical involvement.

There was marked destruction of the renal papilla in the mice that died but this was also seen in some of those which were killed when apparently well and, in a subsequent experiment, some indication was obtained that the lesions, although in typical cases acute and eventually fatal, might become chronic.

No significant changes were seen in the sections of the spleen.

MULTIPLICATION OF *C. renale* IN THE KIDNEY OF THE MOUSE AFTER INTRAVENOUS INOCULATION

In order to satisfy ourselves that multiplication of *C. renale* occurred in the kidney tissue of the mouse after intravenous inoculation, experiments involving actual counts of bacteria were designed. Two experiments were made; in the first, mice were inoculated with saline suspensions prepared from agar slope cultures and calculated to contain approximately 20 million living organisms in each

FIG. 1.—Papilla of kidney, mouse. The tip shows early necrosis, hyaline material, and clumps of organisms. Some of the intertubular capillaries are dilated, and filled with inflammatory cells. H. & E. × 60.

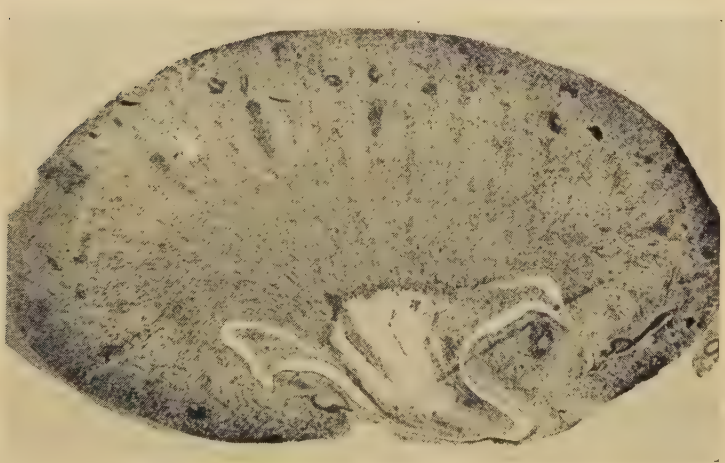

FIG. 2.—Kidney, mouse. The papilla is necrotic, with an upper margin of inflammatory cells. The pelvis is dilated, and contains pus. Intertubular and perivascular foci of mononuclear cells in the cortex are not referable to *C. renale*, as they occur to a varying extent in the kidneys of a proportion of apparently normal uninjected mice. H. & E. × 14.

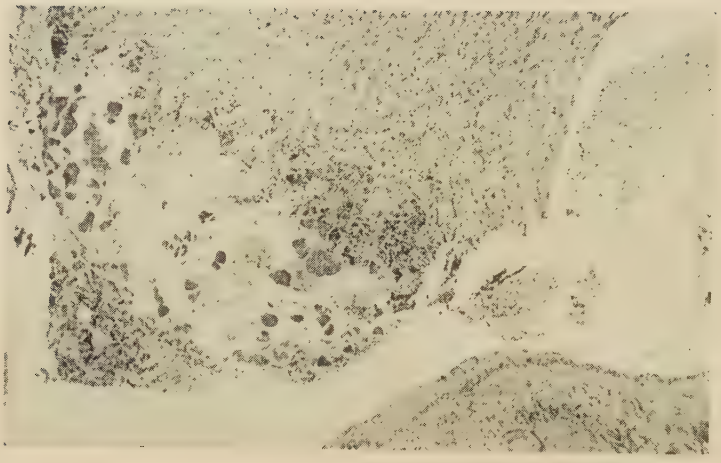

FIG. 3.—Papilla of kidney, mouse. The apex of the papilla shows necrosis, with clumps of organisms, and an upper border of inflammatory cells. Pus is present in the dilated pelvis. H. & E. × 45.

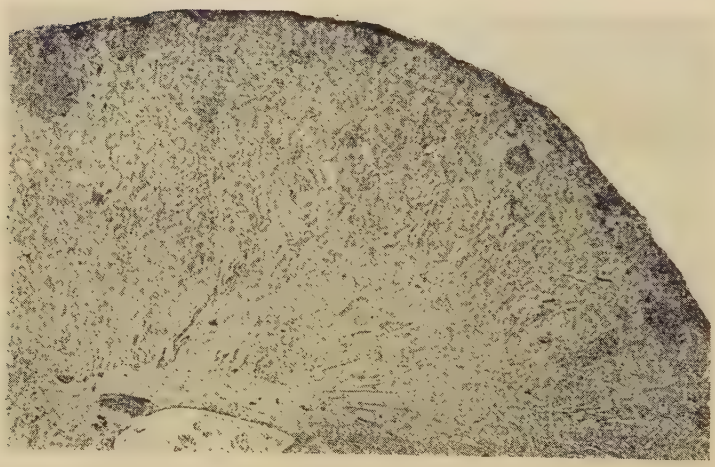

FIG. 4.—Part of kidney, mouse. Showing changes interpreted as due to chronic pyelonephritis, with pus in the pelvis, interstitial infiltration and fibrosis, dilatation or atrophy of tubules, and an irregular surface. Several mononuclear cell foci are present. H. & E. × 25.

dose; in the second experiment mice were given a suspension of bacteria grown in broth for six hours at 37° C. and the actual number of living organisms given to the mice was determined by a variable count. In each experiment groups of three mice were selected at predetermined intervals and these were killed; the kidneys and spleen were removed aseptically and ground up in a measured quantity of saline and counts made of the number of living organisms in the supernatant fluid. In the second experiment the lungs were also examined. Calculations were made of the mean number of living organisms present in the kidneys, spleen and, in the second experiment, in the lungs of mice killed at a particular time. The relevant data are given in Tables III and IV.

TABLE III

CALCULATED NUMBER OF *C. renale* PRESENT IN THE KIDNEYS AND SPLEEN OF MICE KILLED AFTER INTRAVENOUS INOCULATION OF APPROXIMATELY 20 MILLION VIABLE *C. renale*

Interval after inoculation	*Number of viable* C. renale *in*	
	Kidneys	*Spleen*
4 hours	66,000	4,200
24 „	101,000	1,465
2 days	3,266,000	0
3 „	9,235,000	0
5 „	153,000,000	0
7 „	0	0
10 „	0	0

In this experiment there is an increase in the kidneys for whereas a saline suspension of approximately 20 million was given to each mouse the number recoverable from the kidneys of an individual mouse was calculated as 153 million after five days. The mice killed on the seventh and tenth days had apparently removed the bacteria from their tissues. It was decided to repeat this experiment with a young culture and a six hours' broth culture of *C. renale* was used and the bacteria concentrated by centrifugation. The actual dose given to each mouse in the second experiment was computed by a viable count to be 22,800,000 living *C. renale*.

TABLE IV

CALCULATED NUMBER OF *C. renale* PRESENT IN KIDNEYS, SPLEEN AND LUNGS OF MICE KILLED AFTER INTRAVENOUS INOCULATION OF 22,800,000 LIVING *C. renale*

Interval after inoculation	*Number of viable* C. renale *in*		
	Kidneys	*Spleen*	*Lungs*
1 day	6,035,000	1,760	286,000
2 days	117,000	0	300
3 „	7,300,000	0	100
4 „	166,000,000	0	3,700
7 „	110,000,000	0	0
10 „	600,000,000	0	300
14 „	3,300,000,000	0	0
21 „	0	0	0

In this experiment one mouse died on the eighth day and *C. renale* was recovered from the kidneys but not from the spleen and lungs. Only two survivors were available on the 21st day and both showed no signs of infection.

It is clear from this experiment, which was checked by a viable count of the inoculum, that considerable multiplication of *C. renale* can occur in the kidneys of mice after intravenous inoculation. As expected, there is evidence of a clearing mechanism at work during the first two or three days and this is apparently adequate in the spleen and slightly less effective in the lungs. In the kidneys, however, multiplication may occur from about the fourth day and in the second experiment here described reaches a maximum on the 14th day. It is calculated that the mean number of viable *C. renale* in both kidneys of a mouse on that day was 3,300 million.

EFFECT OF INOCULATION OF *C. renale* INTO MICE BY ROUTES OTHER THAN THE INTRAVENOUS ONE AND THE LACK OF LESIONS AFTER THE INTRODUCTION OF SMALL DOSES INTRAVENOUSLY

In order to determine whether localisation of *C. renale* occurred in the kidneys of mice inoculated by other routes, a series of mice were treated as follows: Saline suspensions of *C. renale* were prepared and approximately 14 million organisms were injected into batches of mice. One batch received the injection intravenously, one intraperitoneally, one subcutaneously, and in one, consisting of female mice, the inoculum was introduced into the vagina whilst under nembutal anaesthesia. Two mice from each batch were selected at intervals from three to 20 days later and killed; cultures were made from the kidneys and urine, and sections of kidney and bladder prepared for histological examination. *C. renale* was recovered from three mice only; these had been inoculated intravenously: one was killed on the seventh day and the other two died. The histological examination of the kidneys of the mouse killed on the seventh day showed inflammation of the papilla, a pyelitis and ureteritis, and there was some interstitial spread upwards to the cortex: this is the only one of all the mice injected in the whole series recorded in this paper to show a glomerular abscess in section. One mouse injected intraperitoneally and killed on the seventh day showed a small intratubular focus of polymorphonuclear cells just below the surface epithelium of the renal papillae. *C. renale* was not recovered from the other kidney of this mouse, nor from the urine. Of the two mice which died on the tenth and 14th day after intravenous inoculation *C. renale* was isolated from the urine of one and from the kidney and urine of the other. Both showed inflammation and necrosis of the papilla and pelvis.

This experiment was repeated with a larger dose of approximately 20 million and the results are given in Table V.

C. renale was recovered from the kidneys or urine or both of seven of the ten mice inoculated intravenously and typical lesions

TABLE V

NUMBER OF MICE FROM WHICH *C. renale* WAS RECOVERED AFTER ADMINISTRATION OF LIVING CULTURES BY DIFFERENT ROUTES, AND NUMBER SHOWING LESIONS IN THE KIDNEYS

Interval after inoculation	*Route of administration*															
	Intravenous				*Intraperitoneal*				*Subcutaneous*				*Per vaginam*			
	N.	*Kc.*	*Kl.*	*U.*	*N.*	*Kc.*	*Kl.*	*U.*	*N.*	*Kc.*	*Kl.*	*U.*	*N.*	*Kc.*	*Kl.*	*U.*
7 days	2	2	1	2	2	0	0	0	2	0	0	0	2	0	0	0
14 „	2	0	0	1	2	0	0	0	2	0	0	0	2	0	1	0
21 „	2	2	0	2	2	0	0	0	2	0	0	0	2	0	0	0
28 „	2	1	1	2	2	0	0	0	2	0	0	0	2	0	0	0
35 „	2	0	1	0	2	0	0	0	2	0	0	0	2	0	0	0

N. = Number of mice examined.
Kc. = „ „ from the kidneys of which *C. renale* was recovered.
Kl. = „ „ showing lesions in the kidneys.
U. = „ „ from the urine of which *C. renale* was recovered.

of the papilla and pelvis were observed in the kidneys of three of them. *C. renale* was not recovered from any of the mice inoculated by the other three routes and in one only was there evidence of histological change in the kidney. This mouse, one having *C. renale* administered per vaginam and killed on the 14th day, showed a typical necrosis and inflammation of the papilla and renal pelvis.

The intravenous route appears to be the only one in which a high proportion of the injected animals show lesions in the kidneys. Even so, adequate dosage is essential; in a further experiment about 12,000 living *C. renale* were given intravenously and none of the mice killed at periods up to 12 days showed lesions when examined histologically, and from none was *C. renale* recovered.

ATTEMPTS TO INFECT MICE BY PLACING NORMAL ANIMALS IN CONTACT WITH INFECTED ONES

It was not possible to effect a spread of infection by contact although *C. renale* is present in the urine of some of the infected mice and is therefore presumably excreted on to the litter. An experiment was made whereby 12 mice were injected by the intravenous route and were allowed to mingle with 12 normal animals for 56 days. Of the 12 injected with approximately 20 millions of *C. renale*, two died after two days with *C. renale* in the blood, spleen, kidneys and urine; one which died on the eighth day, one on the 13th day and two on the 19th day contained *C. renale* in the kidneys and urine. *C. renale* was not recovered from two which died on the 40th and 45th days respectively. Of the uninoculated mice, six died and six were killed and *C. renale* was not recovered from them, neither were there any lesions in the urinary tracts when examined histologically. Of the eight inoculated mice which died, seven showed typical histological lesions of varying stages of

development: one mouse which died on the 40th day and was negative by bacteriological examination, showed evidence of a chronic pyelonephritis with fibrosis of the wall of the renal pelvis and patchy atrophy or dilatation of the cortical tubules. Two of the inoculated mice which were killed on the 56th day showed a similar chronic lesion. (See Fig. 4.)

From these and the other examinations it appears as if chronic pyelonephritis following intravenous inoculation of *C. renale* is not usual, an acute condition being commoner.

It is proposed to record at a later date the results of the injection of other species of bacteria and inanimate substances such as indian ink. This comparative study should indicate whether *C. renale* has a specific action.

EFFECTS OF INJECTIONS OF *C. renale* IN GUINEA-PIGS, RABBITS AND THE DEVELOPING EGG

Guinea-pigs were injected intravenously with approximately 60 million living *C. renale* and individual animals were killed from one to 14 days later. *C. renale* was recovered from the spleen and kidneys of one guinea-pig killed one day after inoculation and from one kidney and the urine of the animal killed on the third day. *C. renale* was not isolated from animals killed on the second, fifth, seventh, tenth and 14th days. The histological signs of renal damage were meagre, consisting of a migration of polymorphonuclear cells into the lumen of the tubules of the kidneys and the formation of some fibrous tissue in the papilla with foci of interstitial cell proliferation and focal tubule atrophy or dilatation.

Rabbits were also injected intravenously with about 60 million living *C. renale* and individual animals were killed from the second to the 15th day. *C. renale* was recovered from the urine of the rabbit killed on the fifth day and from the kidney and the urine of the rabbit killed on the 12th day. No *C. renale* was isolated from the rabbits killed on the second, eighth and 15th day. The histological picture of renal damage in this species of animal is also very much less apparent than in the mouse. There appears to be some slight but acute inflammation at the upper level of the papilla by the second day, but these foci may heal. Later the kidneys show small scattered foci of interstitial mononuclear cell proliferation and fibrosis with atrophy or dilatation of the tubules. The rabbits killed on the 12th and 15th day respectively showed in the one a pyelitis with blood and polymorphonuclear cells in the renal pelvis, and in the other there were interstitial foci in the papilla, medulla and cortex containing mononuclear and polymorphonuclear cells.

C. renale was inoculated on to the chorio-allantoic membrane of nine-day-old chick embryos which were examined on the first, second, third and fourth days. *C. renale* was recovered from the allantoic fluid in all cases and from the liver of all but one. One of three eggs examined on the third day and all of those examined

on the fourth day showed a thickened oedematous membrane with a dryish central plaque consisting of a yellow necrotic area. Diphtheroids were seen when the lesions were examined microscopically. There were no apparent lesions in the kidneys. All the embryos examined were alive when the eggs were opened.

DISCUSSION

The development of the lesions in mice after intravenous inoculation of *C. renale* is in some respects similar to that described by Ernst (1905) in cattle which were naturally infected. He studied material collected at slaughterhouses and noted the accumulation of homogeneous material and polymorphonuclear cells in the lumen of the collecting tubules. These may be packed with bacteria eliminated from the labyrinth and give the medullary zone a striated appearance. There is then a disintegration of these tubules which results in a necrosis of the papilla which may be converted to a sequestrum surrounded by healthy tissue; later the necrosed papillae become separated from the parenchyma by fibrous tissue. If the obstruction of the ureters prevents the elimination of the organisms and the flow of urine, a cavernous pyelonephritis results. Further papillae may subsequently become involved but it must be noted that whereas the kidneys of cattle possess multiple papillae, one alone is present in the mouse kidney. By his studies Ernst formed the view that the route of infection was a haematogenous one, although this is not universally accepted.

The studies here recorded suggest that infection by a haematogenous route is possible, although the conditions in the mouse are not necessarily comparable with those in cattle naturally infected. It has been shown that a pyelonephritis may be produced in mice by intravenous inoculation of *C. renale* and that actual multiplication of bacteria occurs in the kidney tissue. These observations, while not necessarily peculiar to *C. renale*, should open up a field for the study of this organism and the diseases it produces.

SUMMARY

The sequence of events occurring in the kidneys which follows the intravenous inoculation of mice with *C. renale* has been studied histologically and bacteriologically.

It has been shown that given in adequate dosage *C. renale* may cause the death of a proportion of mice and induce a pyelonephritis. Similar lesions may be found in some mice which survive.

The changes in the kidneys are associated with actual multiplication of bacteria in the kidney tissue and the typical lesion occurs most frequently in the mice inoculated intravenously.

Other routes of injection were tried and lesions were found in one only of those mice inoculated intraperitoneally, in one only given *C. renale* per vaginam, and in none of those inoculated subcutaneously.

Although it is not suggested that these phenomena are peculiar to *C. renale*, these studies should form a useful basis for an extended investigation into *C. renale* and the disease it produces.

A brief record is given of the effect *C. renale* produces after intravenous inoculation of guinea-pigs and rabbits and of the reaction in the developing egg.

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